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Frank Radella
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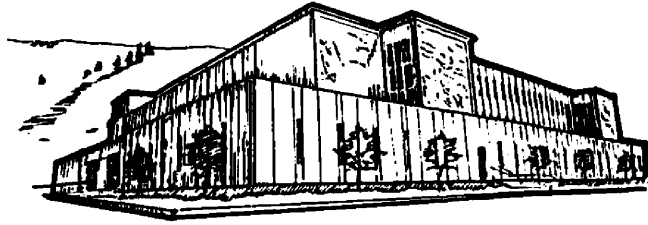
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ANALYSIS OF A 55 KDA PLASMA PROTEIN(S) FROM
BIOMPHALARIA GLABRATA
ASSOCIATED WITH RESISTANCE TO THE HUMAN BLOOD FLUKE
SCHISTOSOMA MANSONI.

by

Frank Radella II

B.S. The University of Montana, 1990

presented in partial fulfillment of the requirements

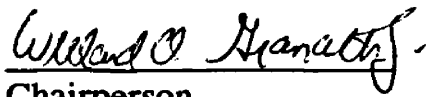
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Analysis of a 55 kDa Plasma Protein(s) from *Biomphalaria glabrata* Associated with Resistance to the Human Blood Fluke *Schistosoma mansoni* (81 pages)

Director: Willard O. Granath, Jr. *WOG*.

Plasma (cell-free hemolymph) factors are involved in resistance of the snail, *Biomphalaria glabrata*, to the human blood fluke, *Schistosoma mansoni*. A 55 kDa polypeptide(s) in plasma from a schistosome-resistant strain of *B. glabrata* (10-R2), but not from schistosome-susceptible snails (M-line), was previously shown to bind to larval *S. mansoni*. This thesis took 5 approaches to further characterize the 55 kDa polypeptide(s) and other plasma factors from both resistant and susceptible strains of *B. glabrata*. The 55 kDa polypeptide(s) from 13-16-R1 (resistant) *B. glabrata* was found to bind to sporocysts when they were alive, but when the sporocysts were chemically-fixed binding of the 55 kDa polypeptide diminished as much as the 210 kDa polypeptide increased. Excretory-secretory products produced by living sporocysts probably cleaves the 55 kDa from the 210 kDa polypeptide. Antibodies generated to the isolated 55 kDa polypeptide(s) from M-line (anti-M-55), 13-16-R1 (anti-R1-55) and 10-R2 (anti-R2-55) *B. glabrata* reacted with the majority of *B. glabrata* plasma proteins. Therefore, antibodies not specific for the 55 kDa polypeptide(s) were immunoprecipitated. The remaining (isolated) antibodies were specific for certain 55 kDa polypeptide(s). Isolated anti-M-55 and anti-R1-55 antibodies reacted stronger to the 55 kDa polypeptide(s) from schistosome-susceptible (M-line) and schistosome-resistant (10-R2) plasma than from schistosome-resistant (13-16-R1) plasma. This suggests that the molecular structures serving as epitopes on the 55 kDa polypeptides(s) are not the same structures which give this polypeptide(s) from schistosome-resistant snails the ability to bind to sporocysts. These and other data suggested that certain plasma proteins possess more than one cross-reactive epitope and these cross-reactive epitopes are more prevalent on certain polypeptides. An immunoprecipitation procedure to remove common antigens between schistosome-susceptible and resistant plasma indicated that anti-M precipitated a 41 kDa polypeptide from M-line but not from 13-16-R1 or 10-R2 *B. glabrata*. Experiments designed to determine the carbohydrate binding specificity of *B. glabrata* plasma proteins indicated that certain proteins were inhibited from binding to zymosan by mannan but the sugar N-acetyl-neuraminic acid caused most *B. glabrata* plasma components to bind to zymosan. Lastly, analysis of *B. glabrata* plasma by two-dimensional electrophoresis indicated that three groups of proteins in the molecular weight range of 55 kDa exist in M-line, 13-16-R1 and 10-R2 *B. glabrata* and all of them are immunogenic. Results of this thesis indicates that *B. glabrata* plasma proteins are similarly immunogenic, but differences in the antigenicity of specific polypeptides exist. These data will be useful in future experiments designed to reveal the significance of the carbohydrate and antigenic nature of plasma polypeptides in immunity of *B. glabrata* to schistosomes.

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Chapter 1

Introduction

Human schistosomiasis is a debilitating disease caused by any one of five digenetic trematodes of the genus *Schistosoma*: *S. mansoni*, *S. haematobium*, *S. japonicum*, *S. intercalatum* and *S. mekongi* (Mahmound, 1992). The World Health Organization estimates that presently at least 200 million people in the world are infected with a schistosome species and approximately 200 thousand people die each year from the disease schistosomiasis (Guyatt and Evans, 1992). *S. mansoni*, the species examined in this thesis, is widespread in tropical and subtropical Africa, South America and the Caribbean (Katz *et al.*, 1989; Mahmound, 1992).

The life cycle of *S. mansoni* is complex. In humans, each sexually mature *S. mansoni* female worm produces approximately 300 fully embryonated (miracidia-containing) eggs each day (Katz *et al.*, 1989). About 50% of the eggs do not exit the host and are swept via the blood stream into the lungs, spleen or liver where the host responds to soluble antigens secreted by the eggs (Katz *et al.*, 1989; Weinstock, 1992). The damaging inflammatory reaction surrounding the eggs trapped in the liver causes hepatosplenomegaly and portal hypertension. These symptoms are responsible for most of the pathology of this disease (Stadecker, 1992). The remaining 50% of the eggs exit the body in feces by working their way, with the aid of histolytic enzymes, through the submucosa and villous tissue (Rollinson and Southgate, 1987). The damage caused by this type of exit results in abdominal pain and diarrhea (Weinstock, 1992). Unfortunately, in developing countries poor sanitation permits the

eggs to reach fresh water (Huang and Manderson, 1992). Once the eggs reach water, changes in osmotic pressure cause them to hatch and the fully embryonated miracidia seek out a snail intermediate host (Jourdane and Theron, 1987). When a suitable snail of the genus *Biomphalaria* is located via chemotaxis the miracidium penetrates the fleshy tissues and rapidly transforms into a germinal sac, termed a mother sporocyst (Noble and Noble, 1982). The fate of the mother sporocyst is dependent upon its genetic makeup and that of the snail (Richards and Merritt, 1972). Some genetically predetermined outcomes for the parasite are: (1) hemocytes (phagocytes) of schistosome-resistant snails recognize, encapsulate, and destroy the larvae; (2) hemocytes from unsuitable snails do not encapsulate the larvae but the parasite does not develop for physiological reasons; (3) the larvae is recognized and encapsulated by hemocytes but the parasite can develop normally; (4) hemocytes do not destroy the parasite and the miracidium develops normally (Bayne *et al.*, 1980b; Richards, 1975a, b; Richards *et al.*, 1992). Several strains of *Biomphalaria glabrata* are available for study including the 10-R2 and 13-16-R1 strains, that are capable of destroying larval *S. mansoni*, and the M-line-strain which allows for normal parasite development (Richards and Merritt, 1972). Once a miracidium has transformed into a mother sporocyst in a susceptible snail (e.g. M-line) the germinal cells lining the inner wall develop into daughter sporocysts which will be liberated when the sac ruptures. The next stage of maturation occurs when the germ cells in the daughter sporocyst develop into cercariae (Noble *et al.*, 1989). Each asexual division results in a large numerical increase of larval parasites. For example, each miracidium can give rise to 4,000

cercariae and up to 40,000 cercariae can develop in an individual snail before the snail becomes overwhelmed with the infection and dies (Katz *et al.*, 1989). In water, cercaria emerge from a snail with the aid of proteolytic enzymes. The cercariae seek out a human, or other definitive host, penetrate a hair follicle and transform to the schistosomule stage in the subcutaneous tissue. After approximately two days in a humans subcutaneous tissue the schistosomule enters the blood stream and migrates to the capillaries of the lung, the heart and then the liver parenchyma where it matures into an adult worm. Once mature, the worms choose their mate for life and pair up with a worm of the opposite sex (Katz, *et al.*, 1989). Once paired, *S. mansoni* worms move to their final destination, the inferior mesenteric veins surrounding the large intestine where the female begins laying approximately 300 eggs each day.

The drugs hycanthone, oxamniquine and praziquantel are useful in treating humans with schistosomiasis. Unfortunately, chemotherapy for control of morbidity and mortality has several limitations: (1) strains of *S. mansoni* that are resistant to hycanthone and oxamniquine have recently been reported (Cioli *et al.*, 1993); (2) drugs are not affordable for most infected people (Presscott, 1987); (3) the prohibitive cost of the skilled manpower required to administer the drugs in endemic countries, (Butterworth *et al.*, 1992); (4) the drug(s) must be administered repeatedly at regular intervals, (Wilkins, 1989); and (5) other drug resistant strains may arise if mass chemotherapy is used (Dias *et al.*, 1982).

Due to these problems additional control measures are often used in conjunction with chemotherapy. Measures to reduce fecal contamination into water have been

attempted (e.g. toilets, water pipe systems and health education). Also, applying molluscicides has had some limited success (Fenwick, 1987; Klumpp and Chu 1987). A problem with these solutions are the high cost and the lack of stable governments (Liese, 1986). Although vaccines may prove valuable in decreasing the prevalence of infection, to date, no vaccine for use in humans exists. An approach that has not yet been attempted is to breed a *B. glabrata* snail strain that is 100% resistant to *S. mansoni* and introduce large numbers into endemic areas (Hubendick, 1958; Jarne *et al.*, 1993; Woodruff, 1978). The snails would act both as a sink for freshly hatched miracidia and, hopefully, out-compete susceptible snails. Focusing on preventing the disease by decreasing the number of vectors could, at very least, decrease the number of worms with which an individual becomes infected. This would be significant since the pathogenicity of the disease is directly correlated with the number of worms a person harbors (Arap *et al.*, 1976; Chen and Mott, 1988; Lehman *et al.*, 1976). An understanding of the immune factors of snails that permit or prevent parasite development will be valuable if snails are to be manipulated for resistance. It would seem necessary to breed a 100% resistant snail strain since flooding an endemic area with snails that are not totally resistance may be counter-productive and result in the introduction of additional genes for susceptibility into the snail population (Richards and Merritt, 1972). In addition, identifying immune factors in the invertebrate may aid in the development of a vaccine for humans (e.g. Capron *et al.*, 1987, 1991, 1992), provide knowledge useful in the development of molluscicides, identify novel

aspects of the vertebrate immune system, and provide a greater understanding of the basic biology of host-parasite immunobiology.

In an effort to elucidate the mechanism(s) of resistance, experiments described in this thesis were performed to identify factors that endow genetically-resistant strains of *B. glabrata* (10-R2, 13-16-R1) with the ability to recognize *S. mansoni* (NIH-Sm-PR-1) sporocysts. Such results were compared to *S. mansoni*-susceptible *B. glabrata* (M-line). This comparison contributes to the identification of factors involved in susceptibility and resistance of these snail strains to the parasite.

1.1. Literature Review

There are three interacting components that determine the fate of a schistosome within a snail: a) snail plasma (cell-free hemolymph) factors, b) snail hemocytes, and c) the larval parasite. Outstanding features of these interactions are that: a) unidentified plasma factors from resistant snails, in some unknown way, enable hemocytes of schistosome-susceptible *B. glabrata* to recognize and become cytotoxic to the parasite (Bayne *et al.* 1980a, b; Granath and Yoshino, 1984); b) hemocytes that recognize the parasite as foreign, encapsulate and destroy it by unknown mechanisms (Loker and Bayne, 1982; Loverde *et al.* 1984; van der Knaap and Loker, 1990), and c) parasite excretory-secretory (ES) products interfere with the normal immune activities of *B. glabrata* (Connors *et al.*, 1991; Lodes and Yoshino, 1990). The following review focuses on how plasma, hemocytes and sporocysts interact.

Plasma from *S. mansoni*-resistant *B. glabrata* can activate hemocytes from schistosome-susceptible snails to kill sporocysts *in vitro* (Bayne *et al.*, 1980b) and *in vivo* (Granath and Yoshino, 1984). In addition to the fact that susceptible hemocytes possess the machinery needed to be cytotoxic (e.g. enzymes/receptors), other information about activation of hemocytes by resistant plasma has been revealed. First, since schistosome-resistant hemocytes are cytotoxic to sporocysts in the presence of schistosome-susceptible snail plasma (Bayne *et al.*, 1980b; Loker and Bayne, 1982), an inhibitory plasma protein that interferes with either a sporocyst or hemocyte receptor is not likely to be responsible for susceptibility of snails to the parasite. Secondly, since schistosome-resistant snail plasma is not cytotoxic to *S. mansoni* sporocysts *in vitro* (Bayne, 1980a; Fryer *et al.*, 1989) the plasma factor(s) that was transferred by Granath and Yoshino (1984) is not an enzyme or other cytotoxic substance that acts directly on the sporocyst. Thus, the transferred factor must have either activated hemocytes or marked the sporocyst for attack (Bayne *et al.* 1985). In an effort to identify the unique plasma factor(s) conferring resistance, Spray and Granath (1988) used sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) to look for differences in plasma between susceptible and resistant snail strains. When the plasma was solubilized without the reducing agent 2-mercaptoethanol the only differences observed were quantitative. The M-line strain also appeared to have more total protein than the 10-R2 strain and this was confirmed using a protein assay. When 2-mercaptoethanol was added to the solubilization buffer a difference in protein migration patterns was observed in the 55-

kDa range in the resistant 10-R2 strain. This indicated a unique disulfide linkage of proteins in this molecular weight region in resistant snails. Loker and Hertel (1987) reasoned that the unique plasma protein(s) might be identified if a snail was exposed to the parasite before analyzing the plasma by SDS-PAGE. These researchers exposed schistosome-susceptible and resistant-*B. glabrata* to the trematode *Echinostoma paraense* eight days before obtaining plasma. Upon performing SDS-PAGE on the plasma from the infected snails the only differences found were quantitative.

Since schistosome-resistant snail hemocytes were cytotoxic in the absence of plasma (Bayne *et al.*, 1980a) a strategy was developed to identify unique proteins synthesized by hemocytes (Yoshino and Lodes, 1988). Many methionine-containing hemocyte proteins that were synthesized *in vitro* in response to schistosome ES products were identified. Lodes and Yoshino (1993) later showed that hemocyte-secreted proteins of 19 and 46 kDa from both resistant and susceptible snails bound to sporocysts *in vitro* but no significant difference in the binding capacity of these proteins between resistant and susceptible snails was found.

Since hemocyte-secreted proteins bind to sporocysts it was suggested that sporocyst binding proteins could be involved in the snails immunorecognition of the parasite (Bayne and Yoshino, 1989). It was also suggested that resistant but not susceptible snails possess a unique agglutinin (Boswell and Bayne, 1985). Therefore, snail plasma was examined for proteins with the ability to bind to sporocysts. When interpreting results of such studies it is necessary to realize that sporocysts live in a

snails hemolymph and, since the parasite contains no digestive system, nutrients are obtained by absorption of certain hemolymph components (Bayne *et al.*, 1985, 1986; Meuleman, 1972). In one *in vitro* experiment, sporocysts acquired various components on the tegument within 20 minutes of being placed in snail plasma. Maximum acquisition of plasma components was obtained at 3 hr, and within 12 hr most plasma components could not be detected using Western blot analysis and SDS-PAGE (Bayne *et al.*, 1986). In the same experiment live sporocysts were shown to acquire the same molecular weight polypeptides between schistosome-resistant and susceptible *B. glabrata* plasma but in different quantities. In other mollusks, various types of hemagglutinins and opsonins have been reported (Anderson and Good, 1976; Arimoto and Trip, 1977; Prowse and Tait, 1969; Renwrantz and Cheng, 1977; Renwrantz *et al.*, 1981). In *B. glabrata*, many hemagglutinins (components which agglutinate red blood cells) and non-hemagglutinins were identified which bound to miracidia, sporocysts and cercariae but none were unique to resistant snails (Couch *et al.*, 1990; Stein and Basch, 1979).

Spray and Granath (1990) used radiolabeled plasma to identify a 55 kDa polypeptide unique to schistosome-resistant *B. glabrata* (10-R2) that adhered to living sporocysts *in vitro*. It was hypothesized that this protein either acted as an opsonin to aid hemocytes in recognizing the parasite or it agglutinated the parasite. Fryer and Bayne (1989) identified a protein in this molecular weight range from resistant (10-R2, 13-16-R1), but not from susceptible (M-line) snails, that acted as an opsonin.

Support for agglutinins in hemocyte-sporocyst interactions comes from Loker *et al.* (1984). These authors observed that glutaraldehyde-fixed sporocysts of *S. mansoni* agglutinated in plasma from schistosome-resistant *B. glabrata* (10-R2 and 13-16-R1), but did not agglutinate in plasma from schistosome-susceptible *B. glabrata* (M-line, MRLc, L-311). In contrast, in this same study living sporocysts agglutinated in both schistosome-resistant and susceptible plasma. This finding lead Spray and Granath (1990) to hypothesize that the unique schistosome-resistant sporocyst-binding 55 kDa polypeptide was the protein responsible for the agglutination of fixed sporocysts. Bayne *et al.* (1986) suggested that acquisition of plasma by the sporocyst modified its tegument. An actual change in the conformation of the plasma component after binding to the parasite could be responsible for the agglutination. Indeed, the 55 kDa protein with the unique disulfide linkage described earlier, could undergo a conformational change when bound to sporocysts which causes sporocysts to agglutinate.

It appears that the 55 kDa plasma polypeptide does not play a role in hemocyte cytotoxicity to sporocysts since schistosome-resistant hemocytes have been shown to be cytotoxic in the absence of plasma factors (Bayne *et al.*, 1980a). Also, the 55 kDa polypeptide was not synthesized by hemocytes (Lodes and Yoshino, 1989; Lodes and Yoshino, 1993) indicating that hemocytes, after encapsulating a sporocyst, do not synthesize and secrete the 55 kDa protein to aid in cytotoxicity.

It may be that agglutination of sporocysts by snail plasma may be a preliminary step for hemocytes to become cytotoxic to the sporocyst (Spray and Granath, 1990).

Hemocytes from susceptible (M-line) *B. glabrata* were shown *in vitro* to be cytotoxic to sporocysts when the sporocysts were pre-incubated in the lectin concanavalin A (Con A) (Boswell and Bayne, 1985). However, when hemocytes were the component pre-incubated in Con A no cytotoxicity occurred to the sporocysts (Boswell and Bayne, 1985; Loker *et al.*, 1989; Yoshino, 1981). This indicated that the components that activate hemocytes were found on the sporocyst surface and not on the hemocyte surface. These results led Boswell and Bayne (1985) to propose that Con A was acting as a bridging molecule between the hemocyte and sporocyst. Indeed, Yoshino (1981) found Con A determinates on *B. glabrata* hemocytes that could link the cells to sporocysts. However, no difference in the molecular composition of the Con A determinates between resistant (10-R2) and susceptible (M-line) *B. glabrata* hemocytes was found.

Much effort has been directed at elucidating the mechanism of sporocyst/Con A activation with the most convincing evidence being presented by Loker *et al.* (1989). These investigators found that Con A modified the sporocyst tegument to enhance hemocyte adherence and cytotoxicity. Tegument modulation was proposed because once Con A was on the sporocyst surface anti-Con A antibodies coated the lectin without inhibiting hemocytes from becoming cytotoxic. This indicated that once the lectin was on the surface of the sporocyst, lectin-hemocyte contact was not required for cytotoxicity to occur. Therefore, the lectin was not serving as a link between the sporocyst and hemocyte. Although Con A *per se* is not present in snail plasma, lectin-like molecules with agglutinating properties were found in *B. glabrata* plasma

(Boswell and Bayne, 1985). Thus it is possible that a plasma component in resistant snails binds to the sporocyst surface to enable hemocytes to become cytotoxic.

To determine if a resistant plasma component first binds to a sporocyst to allow cytotoxicity, sporocysts or susceptible hemocytes were incubated in resistant plasma and then mixed together (Loker and Bayne, 1982). Also, sporocysts, resistant-plasma and susceptible-hemocytes were mixed at the same time. Results of various combinations revealed that susceptible hemocytes were able to inflict damage to sporocysts only when sporocysts, plasma and hemocytes were mixed at the same time. This finding did not support or refute the hypothesis. The resistant plasma component was necessary for cytotoxicity, but it could not be shown to bind to the sporocyst before activating hemocytes to become cytotoxic. This failure may be because living sporocysts or hemocytes pre-incubated in the resistant plasma, process, destroy, shed or internalize the plasma bound to it (Bayne *et al.*, 1985; Yoshino, 1981). It is evident that induced cytotoxicity is complex but it can be studied by asking the following: (1) what is on the sporocyst surface that serves as a receptor for plasma or hemocytes? and (2) how do opsonins aid in recognition of foreign surfaces by hemocytes?

Lectins, substances that recognize specific carbohydrates, have given much information about the chemical composition of possible receptors on the sporocyst surface. A variety of lectins bound to *S. mansoni* and *E. paraensei* sporocysts *in vitro* (Uchikawa and Loker, 1991; Yoshino *et al.*, 1977). Differences in lectins with galactosyl determinants for *in vitro*-transformed mother and daughter sporocysts have

been described (Zelck and Becker, 1990). It is possible that lectins play a role in immuno-recognition of *B. glabrata* since molluscan hemocytes were shown to have lectin binding sites on their surface (Parrinello and Arizza, 1988; Renwranztz and Cheng, 1977; Yoshino, 1983), anti-lectin antibodies bound to hemocytes of the snail *L. stagnalis* (van der Knaap *et al.*, 1981), and a lectin was isolated from oyster hemocyte membranes (Vaste *et al.*, 1982). It is not know whether these hemocyte-associated lectin receptors play a role in resistance to schistosomes. In fact, Yoshino (1981) showed that determinates for Con A from both susceptible and resistant hemocytes were of the same molecular composition.

The means by which hemocytes from the mussel *Mytilus edulis* recognize foreign surfaces *in vitro* was examined by Renwranztz and Stahmer (1983). These investigators showed that yeast pre-incubated in plasma was phagocytosed because of two specific plasma lectins; thus these lectins functioned as opsonins. Also, the addition of Ca^{++} to hemocytes enhanced phagocytosis of saline-suspended yeast which led these researchers to propose that a divalent cation-dependent molecule occurs on the hemocyte surface which activated the cell. Further, the glycoprotein mucin inhibited both the opsonization of yeast cells to the surface of hemocytes and the agglutinating activity of the isolated lectins. This suggested that both the plasma lectins and the molecule on the hemocyte surface had similar recognition sites. In another study, Fryer and Bayne (1989) used an *in vitro* yeast phagocytosis assay to examine the carbohydrate specificity of opsonins for hemocytes of *B. glabrata*. They found that a cell-bound opsonin for the yeast was inhibited by laminarin, but a plasma

opsonin was inhibited by mannan. This indicated that the cell-bound and plasma opsonin recognized different carbohydrates.

The role of plasma agglutinins/lectins emerging thus far is: (1) agglutinins could be opsonic and/or could clump sporocysts to aid in hemocyte recognition; (2) specific exogenous lectins modulate the sporocyst surface to allow cytotoxicity; and (3) opsonic plasma proteins increase phagocytosis of foreign material and these components could either have the same or different carbohydrate recognition sites for foreign particles.

As previously discussed, carbohydrate molecules are very important in immunorecognition in schistosome-snail interactions. These carbohydrates could also be responsible for the cross-reactivity between sporocysts and snail plasma (Dissous *et al.*, 1986). This was based on a study where antibodies generated to deglycosylated plasma did not cross-react with miracidia whereas antibodies to glycosylated plasma did cross-react. In addition, a monoclonal antibody to a 90 kDa glycosylated component did not recognize this component if it was deglycosylated nor any miracidial antigens if they were deglycosylated. In contrast, when the glycoproteins were not deglycosylated the antibody bound both the 90 kDa component and miracidia antigens.

Additional studies on carbohydrate epitopes have shown that mouse monoclonal antibodies, generated to *in vitro* transformed sporocysts, recognized only carbohydrate epitopes on the sporocyst surface (Boswell *et al.*, 1987). Some of these glycoconjugates on the surface of *S. mansoni* sporocysts were pronase-sensitive and

others were pronase-resistant. This indicated that the glycoconjugates were bound to different proteins. Also, endoglycosidase had no effect on the binding of lectins to the surface of living *in vitro*-transformed sporocysts of *S. mansoni* or *E. paraensei* (Uchikawa and Loker, 1991) indicating that the sugar residues were not cleaved by this enzyme. Since the parasite was alive this study, the metabolically active tegument could have either destroyed the enzymes or produced more of the sugars against which the enzymes were targeted.

Numerous receptors that are most likely carbohydrates are present on the sporocyst surface (Bayne *et al.*, 1984). These investigators showed that equivocal amounts of two anti-sporocyst antibodies (IgG), with some overlapping specificities for the sporocyst tegument, differentially affected cytoadherence (CA) of hemocytes. This finding indicated that sporocysts have numerous receptors on their surface involved with CA. These results occurred with both fixed and living sporocysts indicating that the IgG was not modulating the sporocyst surface to allow CA. Also, the F(ab')₂ from these antibodies affected CA in the same manner as did the intact IgG molecule showing that interference caused by the intact antibody was not due to steric hinderance.

Encapsulating a sporocyst probably is an important preliminary step for cytoadherence. Although both resistant and susceptible *B. glabrata* hemocytes encapsulated sporocysts *in vitro* (Bayne *et al.*, 1984), when hemocytes from schistosome-resistant snails encapsulated sporocysts, portions of the tegument was phagocytosed (Lie, 1982; Lie *et al.*, 1987; Loker and Bayne, 1982), cytotoxicity was

observed, and the sporocyst was killed (Bayne *et al.*, 1980b; Lo Verde *et al.*, 1984). Conversely, hemocytes from schistosome-susceptible M-line *B. glabrata* encapsulated *S. mansoni* sporocysts without damaging the sporocyst (Bayne *et al.*, 1980b, 1984; Loker *et al.*, 1989). This observation indicated that more than just encapsulation of sporocysts was involved with parasite destruction.

How *B. glabrata* hemocytes kill a sporocyst is unknown, but many cytotoxic enzymes have been associated with the process. For example, alkaline phosphatase, aminopeptidases, lysozyme and acid phosphatase levels increase in snail hemolymph in response to infection (Cheng, 1978; Cheng and Butler, 1979; Kassim and Richards, 1978). Acid phosphatase was also detected around degenerating encapsulated sporocysts (Cheng and Garrabrant, 1977). Using three lysosomal markers, acid phosphatase, peroxidase and non-specific esterase, Granath and Yoshino (1983) found that hemocytes from resistant (10-R2) *B. glabrata* had a greater distribution and abundance of these enzymes than hemocytes from susceptible (M-line) *B. glabrata*, and each strain had discrete hemocyte subpopulations based on these markers. Bayne *et al.* (1980b) suggested that hemocytes from resistant *B. glabrata* (10-R2, 13-16-R1) exist in a naturally higher state of cytotoxic activation than susceptible (M-line) *B. glabrata*. Although the mechanism of cytotoxicity is unknown, it could be related to the production of reactive forms of oxygen (e.g. O_2^- , H_2O_2) as was demonstrated *in vitro* by several groups (Connors *et al.*, 1991; Dikkeboom *et al.*, 1988; Shozawa *et al.*, 1989; van der Knaap and Loker, 1990).

If snails produce toxic molecules then a sporocyst must prevent or inactivate these molecules to survive. Indeed, *in vitro* and *in vivo* studies with hemocytes from *B. glabrata* infected with *E. paraensei* showed that the hemocytes had a reduced ability to encapsulate and destroy *S. mansoni* larvae or foreign particles (Lie, 1982; Lie *et al.*, 1987; Lie and Heyneman, 1977; Loker *et al.*, 1986; Noda and Loker, 1989). To determine if the interference was acting on plasma or hemocytes, Noda and Loker (1989) assayed phagocytosis by *B. glabrata* hemocytes incubated in homologous or heterologous plasma from uninfected and *E. paraensei*-infected snails. Their results revealed that hemocytes from uninfected snails consistently had elevated levels of phagocytosis compared to hemocytes from infected snails. These results were interpreted to mean that the essential regulating factor in phagocytosis was the specific source of hemocytes rather than the plasma.

Noda and Loker (1989) observed that ES products of *E. paraensei* excreted *in vivo* had no inhibitory effect on phagocytosis by hemocytes *in vitro*. This was revealed when plasma from M-line *B. glabrata*, infected for eight days with *E. paraensei*, was used to pretreat sheep red blood cells (sRBC) and no inhibition of phagocytosis was observed. Although specific ES products had been shown to specifically interfere with hemocyte motility (Lodes and Yoshino, 1990) it appeared that they did not affect hemocyte phagocytosis of a foreign particle pre-incubated in ES products excreted *in vivo*. Also, the lack of inhibition could be a result of the *in vitro* phagocytosis assay of a biologically irrelevant particle (sRBC) that did not allow ES products to perform their normal *in vivo* function, or the ES products had been

destroyed by the immune system of the snail. Hemocytes from *E. paraensei*-infected snails capable of phagocytosing sRBC corroborates Lie *et al.* (1981) who showed that *B. glabrata* still retained the ability to phagocytose foreign particles when infected with echinostomes. Lie *et al.* (1981) also showed that an echinostome infection caused *B. glabrata* to lose the ability to encapsulate different species of digenean larvae. This indicated that the interference of the infecting echinostome was specific.

A 108 kDa ES protein from *S. mansoni* that had specific interfering abilities was isolated by Lodes and Yoshino (1990). These investigators showed, *in vitro*, that this 108 kDa ES protein significantly inhibited the random motility of M-line, but not 10-R2, hemocytes. In addition, Connors and Yoshino (1990) showed that resistant 10-R2 hemocytes maintained a higher degree of phagocytosis and superoxide production than did susceptible M-line hemocytes in response to sporocyst ES products. Also, ES products were shown to inhibit hemocyte protein synthesis (Lodes *et al.*, 1991).

1.2. Overall Objectives

From the above, it is clear that plasma factors play a significant role in sporocyst/hemocyte interactions. The research described in this thesis was based largely on the studies of Spray and Granath (1990) who identified a 55 kDa plasma protein from schistosome-resistant *B. glabrata* (10-R2) that bound to living sporocysts. This thesis focused on the characterization of the sporocyst and zymosan-carbohydrate binding abilities, and the antigenic properties of the 55 kDa plasma protein(s) from *B. glabrata*. In addition, immunoprecipitation and two-dimensional electrophoresis were techniques used to identify unique antigens in *B. glabrata* plasma

since unidentified plasma components from *S. mansoni* resistant snails enabled *S. mansoni*-susceptible hemocytes to become cytotoxic to sporocysts *in vivo* (Granath and Yoshino, 1984).

1.3. Specific Research Goals:

1. To determine which ^{125}I -labeled plasma proteins from *B. glabrata* (13-16-R1) bind to *in vitro*-transformed living and chemically-fixed sporocysts.
2. To characterize the 55 kDa protein(s) present in M-line, 13-16-R1 and 10-R2 strains of *B. glabrata*. The 55 kDa protein from each strain was used to generate antibodies in rabbits. Each of the rabbit sera containing the anti-55 kDa antibody was used in Western blots to probe plasma from M-line, 10-R2, and 13-16-R1 *B. glabrata* to determine if the antibody was specific for the 55 kDa protein against which it was generated, or if specific strain differences could be detected.
3. To examine the effect carbohydrates have on altering the ability of snail plasma components to bind to zymosan, a particle routinely used to investigate phagocytosis by invertebrate hemocytes.
4. To immunoprecipitate common plasma antigens between schistosome-susceptible *B. glabrata* (M-line) and schistosome-resistant *B. glabrata* (13-16-R1, 10-R2) by using an antibody created against M-line plasma. Plasma antigens not removed by the antibody should be unique to schistosome-resistant snails.
5. To determine if the 55 kDa polypeptide dissociated to smaller molecular weight polypeptides by two-dimensional SDS-PAGE. This will show if the 55 kDa breaks down to smaller molecular weight polypeptides.

6. Two-dimensional electrophoretic separations of M-line, 13-16-R1 and 10-R2 *B. glabrata* plasma were probed with antibodies to plasma from these 3 strains of snails to determine whether the 55 kDa or any other polypeptide was unique to schistosome-resistant snails.

Chapter 2

Materials and Methods

2.1. Maintenance of *B. glabrata* and Plasma Preparation.

The M-line (schistosome-susceptible), and the 13-16-R1 and 10-R2 (schistosome-resistant) strains of *B. glabrata* used in this study were maintained in continuous culture in 40 liter aquaria that received constant filtration and aeration. Snails were fed leaf lettuce *ad libitum* and were maintained at 26°C in the dark. Hemolymph was collected by headfoot puncture (Sminia and Barendsen, 1980) and placed in a glass depression slide in a humidified chamber for 1 hr to allow hemocytes to adhere to the glass. Plasma was placed in 1.5 ml microfuge tubes, centrifuged for 10 min at 1,000g to pellet any remaining hemocytes, transferred to a new tube, and centrifuged at 10,000g for 10 min to remove remaining particles. The plasma was used immediately or frozen at -20°C.

2.2. Maintenance and Collection of *S. mansoni*.

Miracidia of *S. mansoni* (NIH-Sm-PR-1 strain) were collected (Yoshino *et al.*, 1977) and transformed to sporocysts *in vitro* using RPMI tissue culture medium (Samuelson *et al.*, 1984) devoid of fetal calf serum. Sporocysts were washed (5X) in phosphate-buffered saline (sPBS) isotonic with *B. glabrata* (Yoshino, 1981). If sporocysts were not used immediately they were fixed for 20 min in a 0.1% glutaraldehyde solution, washed (5X) with Chernins balanced salt solution (CBSS) (Chernin, 1963), washed (5X) with sPBS and stored in a 0.02% sodium azide-sPBS solution at 4°C.

2.3. Radioiodination of Plasma.

Plasma was labeled by placing 200 μ l into an Iodogen (1,3,4,6-tetrachloro-3 α ,6 α -diphenylglycouril)-coated 1.5 ml microfuge tube. Iodogen tubes were prepared by placing 20 μ l of a 1.0 mg/ml solution of Iodogen in chloroform into microfuge tubes and allowing them to air dry. Next, 4.0 μ l of 125 I (ICN Pharmaceuticals, Irvine, CA) (25 μ Ci/ μ l, 100 μ Ci total) was added and incubated on ice for 1 hr (Judd, 1987). The resulting radiolabeled plasma was used in experiments described below. Methods described by Spray and Granath (1990) were used to determine which radiolabeled plasma polypeptides from 13-16-R1 *B. glabrata* bound to fixed *S. mansoni* sporocysts.

2.4. Isolation of the 55 kDa Polypeptide(s).

Plasma was separated using preparative SDS-PAGE using the Tris glycine system of Laemmli (1970). To isolate the 55 kDa protein, 10% (acrylamide: N',N'-methylene-bis-acrylamide ratio, 30:0.8) slab gels with a 2.5% acrylamide stacking gel was used. The 55 kDa band was excised with a razor blade and further purified using a 12.5% SDS-polyacrylamide gel. The gel was electroblotted onto a nitrocellulose or a nylon membrane using a transblot chamber (20 mM phosphate buffer pH 0.4; 0.5 A for 18 hrs at constant 15 V). Following Coomassie Brilliant Blue (CBB) staining of the membrane the 55 kDa band was cut out and placed into a 1.5 ml microfuge tube. The procedure of Judd (1988) was used to remove the protein from the membrane.

2.5. Two-Dimensional SDS-PAGE.

The 55 kDa polypeptide from M-line, 13-16-R1 and 10-R2 was excised from a SDS-polyacrylamide gel (10%), solubilized for 10 min and re-subjected to SDS-PAGE (10%) as described by Judd (1988). When solubilized the second time a 1:1 ratio of solubilization buffer to protein was used.

2.6. Generation of Antibodies.

Antibodies to the 55 kDa protein(s) from 10-R2 *B. glabrata* were made by mixing the protein in mammalian PBS (mPBS) with Freund's incomplete adjuvant, (1:1) and injecting 0.5 ml subcutaneously into the axilla and inguinal areas of rabbits (total of 2.0 ml injected). At 1 week intervals 2.0 ml of the 55 kDa antigen in mPBS was injected intravenously. Starting at one month after initial injection blood was collected from the rabbits. The titer of antibodies made with Freund's incomplete adjuvant was less than 10 so a RIBI adjuvant (synthetic trehalose dicorynomycolate (S-TDCM)) (RIBI immunochem, Hamilton, MT) was used and the recommended protocols were followed. Using this protocol, antibodies were created to the 55 kDa polypeptide from M-line (anti-M-55), 13-16-R1 (anti-R1-55) and 10-R2 (anti-R2-55) *B. glabrata*. Rabbit serum was collected and stored using standard procedures. Antibodies were also generated to whole plasma from M-line (anti-M), 13-16-R1 (anti-R1) and 10-R2 (anti-R2) *B. glabrata*.

2.7. Immunoblotting.

Samples of plasma, plasma plus sporocysts or isolated 55 kDa protein were separated by SDS-PAGE and electroblotted onto membranes. Membranes were

blocked with PBS containing 0.5% Tween and 2% milk for 1 hr. Antibodies were initially diluted in the blocking agent at a 1:100 dilution and incubated with a membrane for 1 hr. The membrane was washed 4X for 1 hr to remove unbound serum and then protein A-peroxidase was added (1:1000) in blocking agent to allow binding to antigen-antibody complexes. These complexes were detected using a developer (60 mg 4-chloro-1-naphthol, 20 ml methanol, 100 ml 20 mM Tris 500 mM NaCl, pH 7.2, and 100 μ l H₂O₂). One hundred microliters of India ink in 25 ml dH₂O was used to stain the membrane to reveal molecular weight markers and confirm the transfer of proteins.

2.8. Removal of Cross-Reactivity.

Preparative SDS-PAGE was used to separate 400 μ l of plasma, prestained molecular weight markers were at each end of the gel. The gel was blotted onto a nitrocellulose or nylon membrane and the membrane was blocked with 0.5% tween-2% milk for 1 hr. Strips were cut from the left and right edges of the membrane, stained with Coomassie Brilliant Blue (CBB) and laid next to the main portion of the membrane to locate the 55 kDa protein. Once located, the 55 kDa region was excised from the main portion of the membrane. This membrane, containing all *B. glabrata* plasma proteins except the 55 kDa protein, was incubated in anti-55 kDa antibodies to remove all antibodies not specific for the 55 kDa protein. Homologous combinations of preparative membrane and antibody were used; for example, if the membrane contained M-line plasma it was incubated in a dilution of anti-55-M. A dilution of the remaining antibodies were tested to determine if they reacted with *B. glabrata*

proteins other than the 55 kDa polypeptide. If cross-reacting antibodies remained the procedure was repeated.

2.9. Protein Determination.

A 60 second microwave bicinchoninic acid (BCA) assay was used to measure the concentration of protein samples (Akins, 1992).

2.10. Immunoprecipitation.

Two-hundred microliters of ^{125}I -labeled plasma was added to an equal protein concentration of anti-M-line antibodies. The quantity of protein in each plasma sample was based on values obtained by Spray and Granath (1988). The plasma-antibody mixture was incubated for 1 hr with agitation at room temp (25°C). The appropriate quantity of protein A sepharose, as recommended by the manufacture, was added and mixed for 1 hr on a rocker. The antibody/antigen complexes were pelleted by centrifugation at 10,000g for 5 min. The supernatants should have contained plasma antigens not removed by the protein A-antibody/antigen precipitation. These supernatant samples were characterized by SDS-PAGE and silver staining. Upon first analysis, most proteins were not removed by the antibody so the same amount of antibody was added to the remaining unsolubilized supernatant and the incubations and precipitations were repeated. On the second analysis 1.5 as much of the M-line supernatant than the 13-16-R1 and 10-R2 samples were loaded onto a gel to clearly illustrate the absence of the 41 kDa polypeptide.

2.11. Zymosan/Plasma Incubation Experiments.

After bleeding snails, 100 μ l of plasma was incubated with 1% w/v of a specific sugar for 1 hr. Sugars tested included glucose, mannan, N-acetyl-neuraminic acid and glucosamine. Next, a 100 μ l suspension of zymosan (yeast cell wall) was added to the plasma and incubated for 1 hr to allow plasma components to adhere followed by washing (4X) in CBSS to remove unbound plasma. Washing consisted of adding 500 μ l of CBSS, vortexing, and centrifuging at 2,000 rpm for 2 min to pellet the zymosan. The supernatant was discarded and fresh CBSS added; washing steps were repeated 4X. Solubilization buffer was added to the zymosan pellet and the plasma components that had bound were detected by SDS-PAGE.

2.12. Two-Dimensional Electrophoresis.

This procedure was conducted using methods described by BioRad (Hercules, CA) with the following modifications. All reagents used were put through a 0.45 μ l filter. Acylamide was stored at 4°C in a dark bottle. CalBioChem protein grade detergent was used without diluting 1:10. Plasma proteins were solubilized in lysis buffer for a minimum of 2 hr at room temperature. Fifty μ l of plasma [10mg/ml] was sufficiently solubilized in 125 μ l freshly made lysis buffer. After solubilized, samples were ultracentrifuged at 100,000g overnight (minimum 8 hr). Prefocusing of gels was not conducted. Tube gels were equilibrated for 5 min in SDS sample buffer prior to running the second dimension. Gels were silver stained as described by Dubray and Bezard (1982).

Chapter 3

Results

3.1. Radioiodination/Incubation Experiments.

The results of incubating ^{125}I -labeled plasma from 13-16-R1 *B. glabrata* with living or fixed sporocysts showed that both groups of sporocysts bound plasma polypeptides of 210, 66 and 55 kDa (Fig. 1). Although there were no qualitative differences in polypeptide acquisition between living and fixed sporocysts quantitative differences were found. A 210 kDa polypeptide bound in much greater quantities to fixed compared to living sporocysts. Accompanying the 210 kDa polypeptides increased binding to fixed sporocysts was a proportionate decrease in binding of a 55 kDa polypeptide.

3.2. Two-Dimensional SDS-PAGE.

When the 55 kDa polypeptide from M-line, 13-16-R1 and 10-R2 was excised from a polyacrylamide gel, re-solubilized, and re-subjected to SDS-PAGE it apparently disassociated into many smaller molecular weight polypeptides (Fig.2). The majority of the 55 kDa polypeptide did remain at the same molecular weight. No polypeptide disassociation products below the approximate molecular weight of 30 kDa were detected.

3.3. Immunoblotting.

Immunoblot analysis using anti-M-55 indicated that these antibodies cross-reacted with most of the polypeptides from all *B. glabrata* strains (Fig. 3A). During the process of removing cross-reactivity, certain polypeptides (e.g. 47 and 84 kDa

regions) were recognized less or no longer reacted with the anti-M-55 antibodies (Fig. 3B). The 47 and 84 kDa regions also were the first regions not recognized by anti-R1-55 and anti-R2-55 after removing cross-reactivity (Data not shown).

When cross-reactivity was removed from anti-R1-55 (Fig. 4B) the antibodies reacted much stronger to the 55 kDa polypeptide(s) from M-line and 10-R2 plasma than the 55 kDa plasma polypeptide(s) from 13-16-R1 *B. glabrata*.

3.4 Immunoprecipitation.

Results of immunoprecipitation to remove common antigens between schistosome-susceptible and resistant snails indicated that anti-M precipitated a 41 kDa polypeptide from M-line plasma, but not from 13-16-R1 or 10-R2 *B. glabrata* plasma (Fig. 5). The concentration of the 41 kDa protein normally present in M-line, 13-16-R1 and 10-R2 *B. glabrata* plasma was shown in the control plasma lanes (Fig. 5). Most proteins in *B. glabrata* plasma were not removed by anti-M (Fig. 5).

3.5 Zymosan/Plasma Incubation Experiments.

Certain sugars altered the binding of specific plasma components to zymosan (Fig. 6). Alterations caused by a sugar was detected by comparing the polypeptide profile when a sugar was present (GA, N, GL, M) to when the sugar was absent (plasma + zymosan, (PZ)) (Fig. 6). N-acetyl-neuraminic acid increased the binding of polypeptides in the range of 25 to 210 kDa. Also, N-acetyl-neuraminic acid allowed components less than 55 kDa to bind to zymosan particles. When plasma was preincubated with mannan before being added to zymosan, most plasma polypeptides did not bind with the absence of hemoglobin being the most obvious.

Figure 1. Autoradiogram of ^{125}I -labeled plasma from 13-16-R1 *B. glabrata* that bound to living (L) and glutaraldehyde-fixed (F) *S. mansoni* sporocysts. Note band at 55 kDa that bound more intensely to living sporocysts and a band at 210 kDa that bound more intensely to fixed sporocysts. Molecular weight markers are in kilodaltons.

L

F

2 0 5-

1 1 6-

9 2-

6 6-

4 5-

3 1-

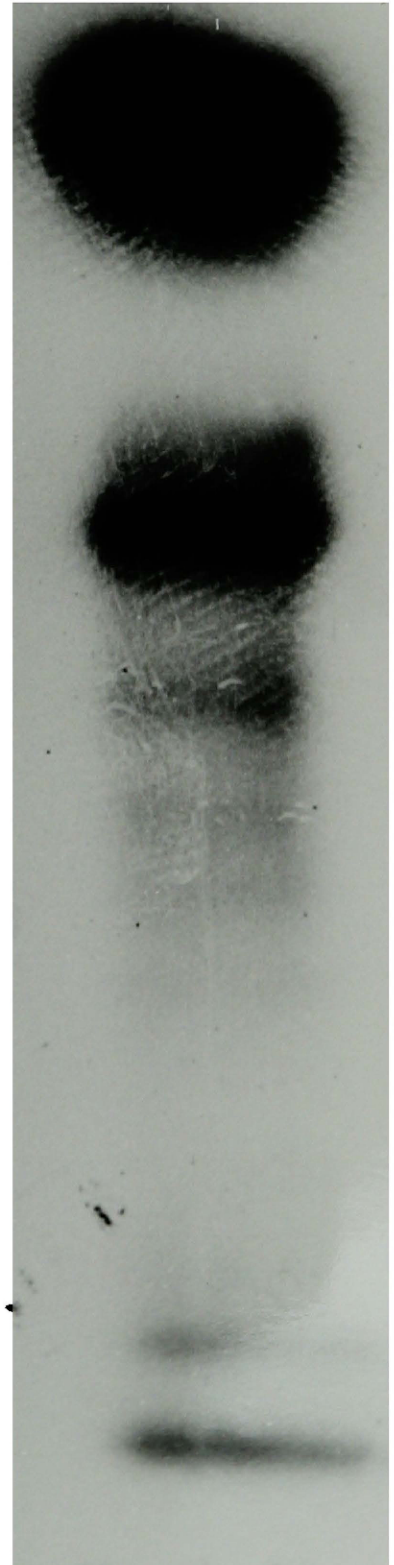


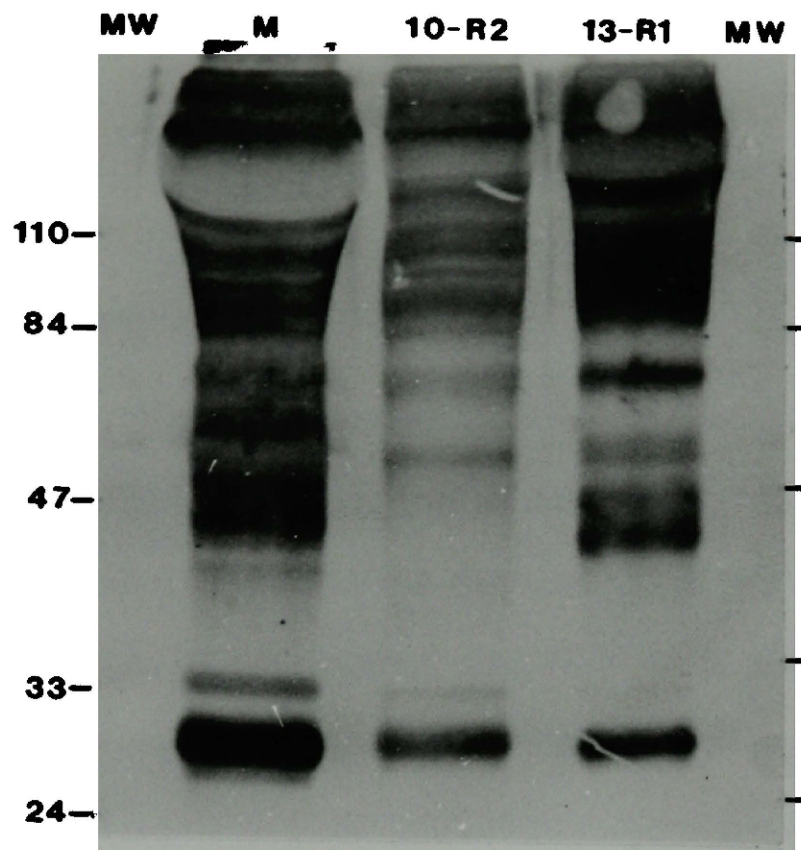
Figure 2. The 55 kDa polypeptide(s) from 13-16-R1, 10-R2 and M-line *B. glabrata* plasma that was excised from a SDS-polyacrylamide gel (10%), solubilized for 10 min and re-subjected to SDS-PAGE (10%). Note the 55 kDa protein(s) disassociated to smaller molecular weight polypeptides. Gel was stained with silver nitrate. Molecular weight markers (MW) are in kilodaltons.

MW 13-16-R1 10-R2 M-Line



Figure 3. Immunoblots of plasma from M-line (M), 10-R2, and 13-16-R1 (13-R1) *B. glabrata* probed with various antibodies. A. Blot was probed with antibodies to a 55 kDa plasma polypeptide(s) from M-line *B. glabrata* (anti-M-55). Note that antibodies to a purified 55 kDa polypeptide(s) from M-line *B. glabrata* reacted with the majority of *B. glabrata* plasma proteins. B. Blot was probed with anti-M-55 which had some cross-reactivity with other *B. glabrata* plasma proteins removed. Cross-reactivity was attempted to be removed by incubating anti-M-55 with all M-line *B. glabrata* plasma proteins except the 55 kDa polypeptide(s). Note that while removing cross-reactivity, proteins in the 47 and 84 kDa regions from three strains of *B. glabrata* plasma were not recognized by the remaining antibodies. Molecular weight (MW) markers are in kilodaltons.

A.



B.

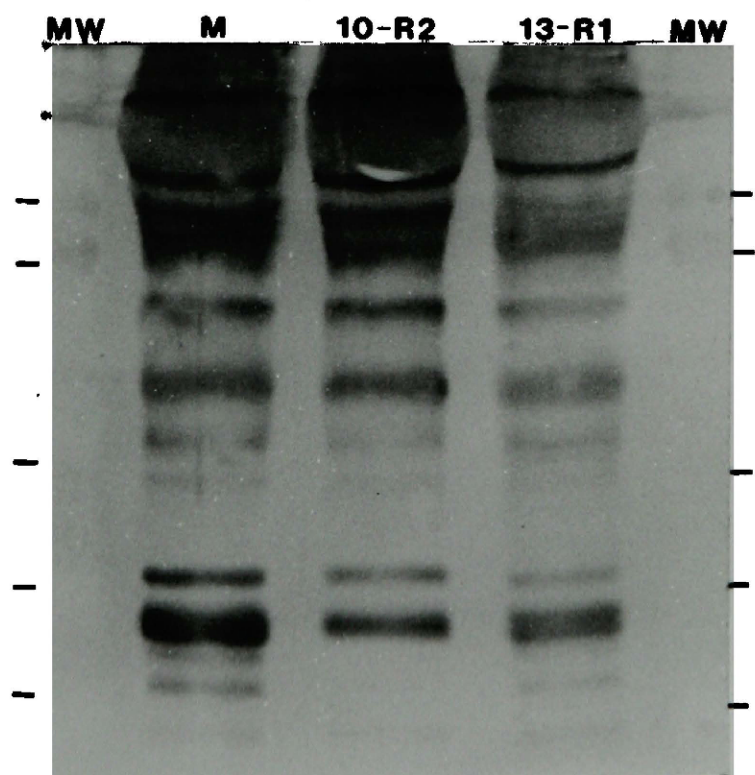
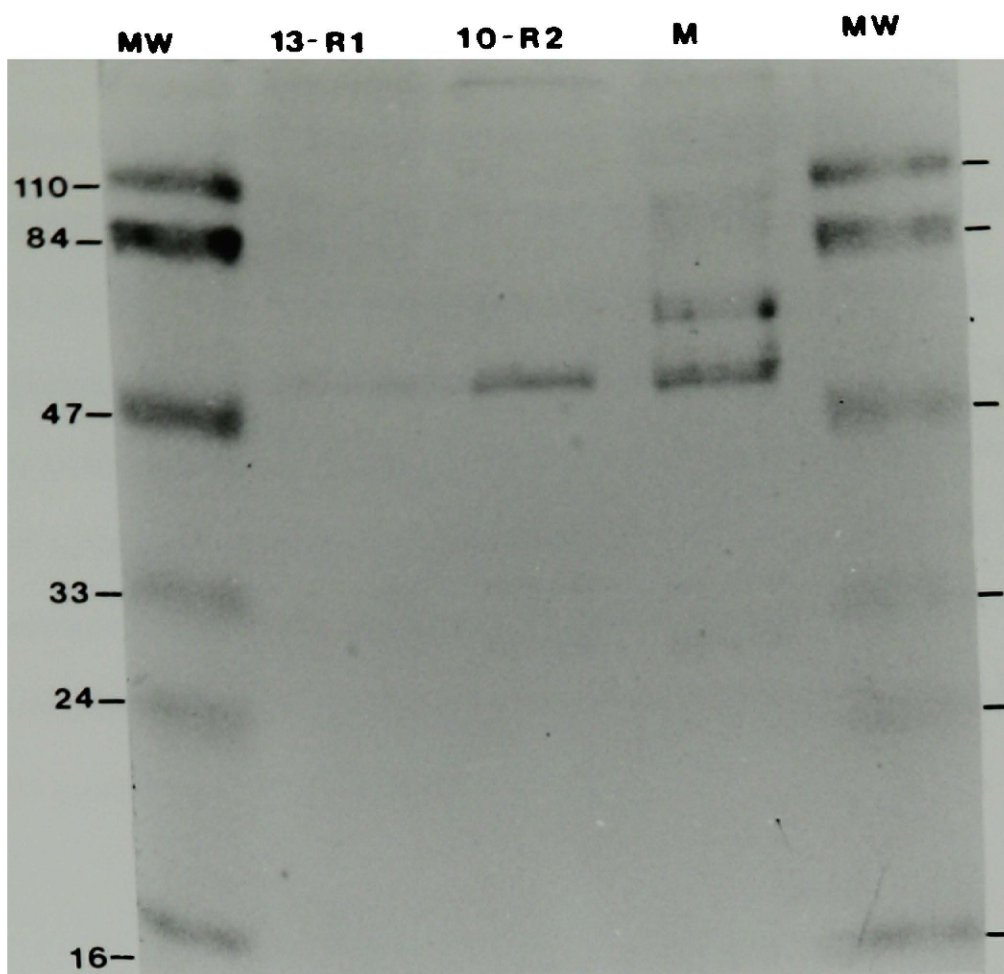


Figure 4. Immunoblots of plasma from 13-16-R1 (13-R1), 10-R2, and M-line (M) *B. glabrata* probed with various antibodies. A. Blot was probed with anti-M-55 which had most of its cross-reactivity with other *B. glabrata* plasma proteins removed. Cross-reactivity was removed by incubating anti-M-55 with all M-line *B. glabrata* plasma proteins except the 55 kDa polypeptide(s). After cross-reactivity was removed from anti-M-55 the remaining antibodies reacted stronger to the 55 kDa protein(s) from M-line and 10-R2 than from 13-16-R1 *B. glabrata*. B. Blot was probed with antibodies raised to a 55 kDa plasma protein(s) from 13-16-R1 *B. glabrata* (anti-R1-55) which had most of its cross-reactivity removed. Cross-reactivity was removed by incubating anti-R1-55 with all 13-16-R1 *B. glabrata* plasma proteins except the 55 kDa polypeptide(s). After cross-reactivity with other *B. glabrata* proteins was removed the anti-R1-55 antibodies reacted stronger to the 55 kDa protein(s) from M-line and 10-R2 than from 13-16-R1 *B. glabrata*. Molecular weight (MW) markers are in kilodaltons.



B.

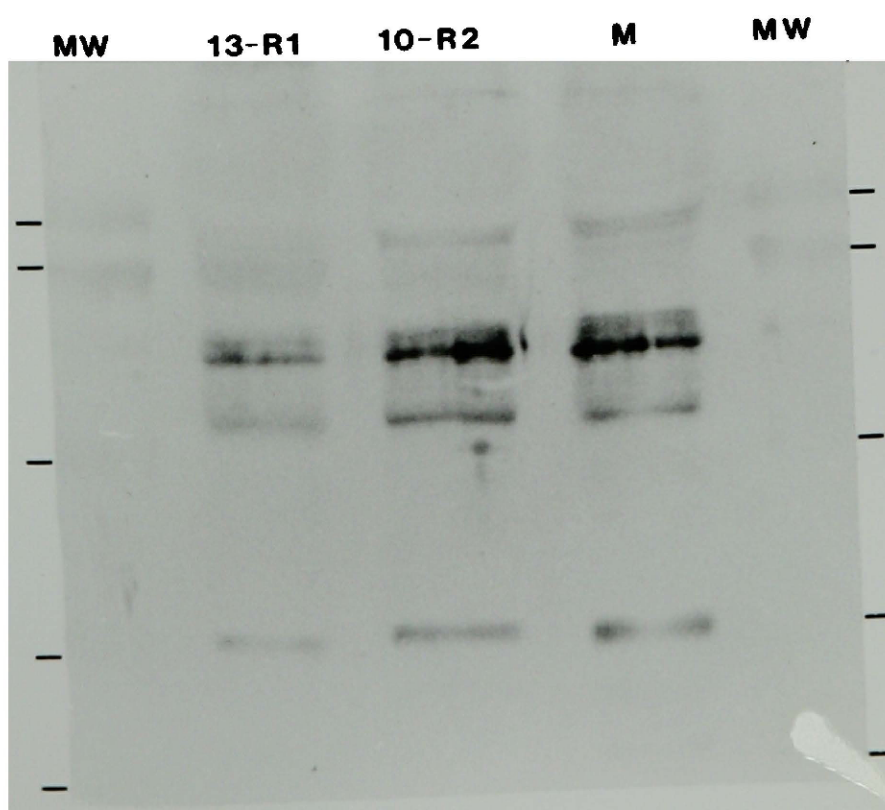


Figure 5. Polypeptide profile of samples separated by SDS-PAGE (10%). Samples included: supernatants (SUP) remaining after immunoprecipitation of plasma from *B. glabrata* 13-16-R1 (13-R1), 10-R2 and M-line (M) with antibody to M-line (anti-M); *B. glabrata* plasma (PLA); anti-M antibodies (AB); protein A (PRA). Protein A was used to pellet (AB)/plasma complexes. Note that a 41 kDa protein was removed from the plasma of schistosome-susceptible snails (M-line) but was not removed from the plasma of resistant snails. This unique plasma protein from 10-R2 and 13-16-R1 *B. glabrata* is indicated with a star. The gel was stained with silver nitrate. Molecular weight (MW) markers are in kilodaltons.

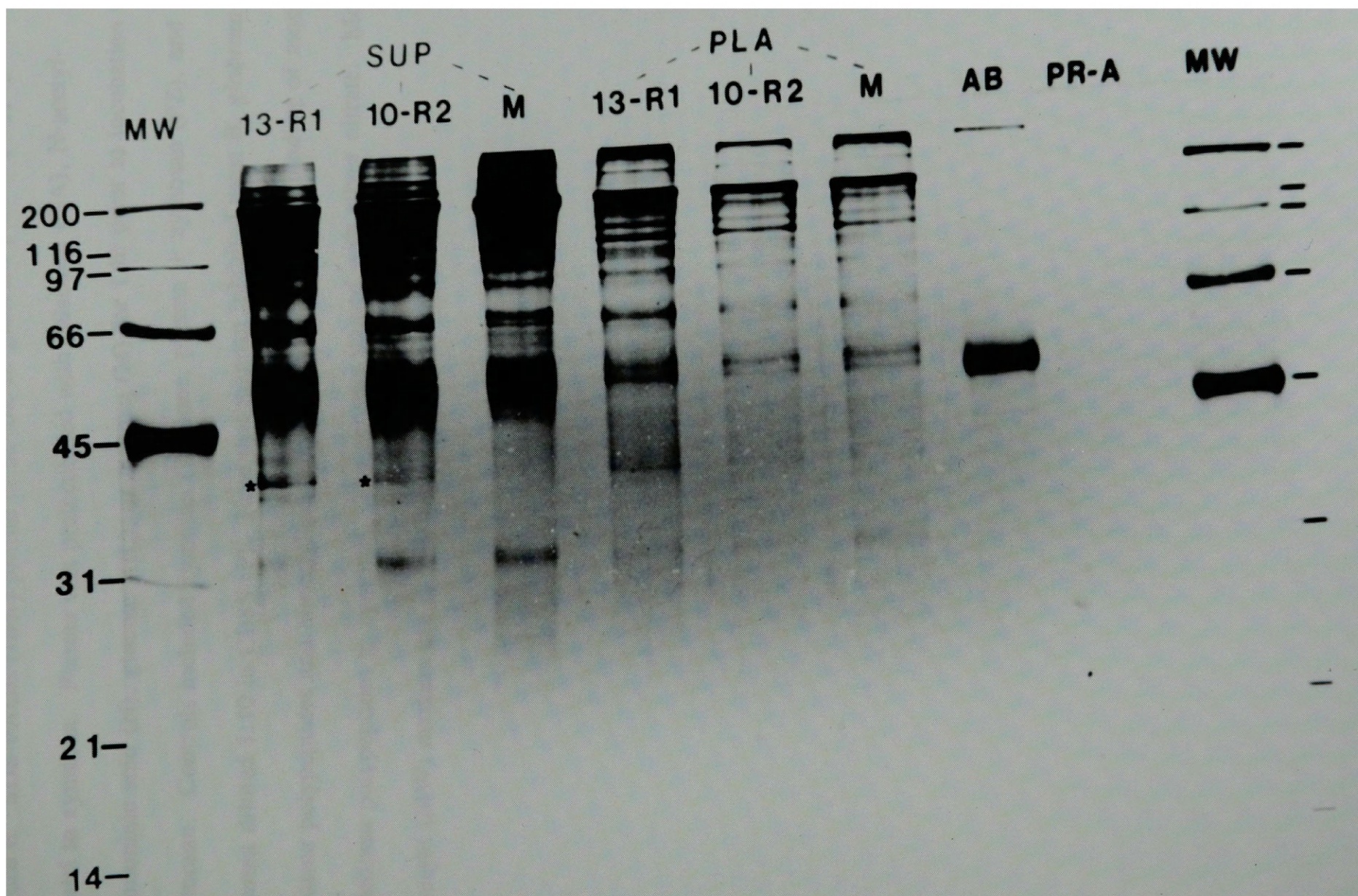
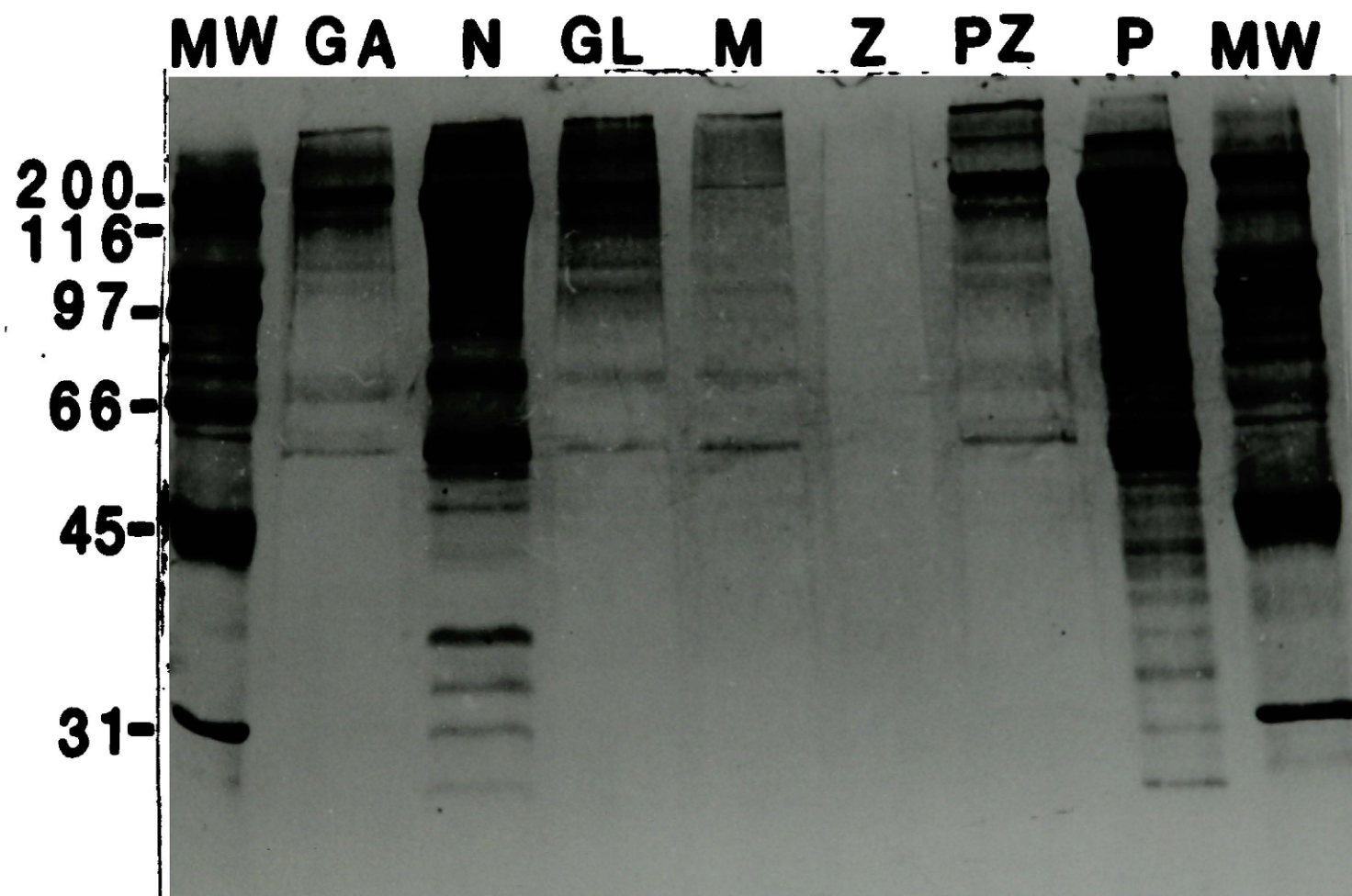


Figure 6. SDS-PAGE (10%) profile of M-line *B. glabrata* plasma polypeptides that bound to zymosan. Plasma was preincubated with galactose (GA), N-acetyl-neuraminic acid (N), glucose (GL), or mannan (M) for 1 hr prior to incubation with zymosan. Controls included zymosan (Z) alone, plasma + zymosan (PZ), and plasma diluted 1:10 in CBSS (P). Note that mannan inhibited most *B. glabrata* plasma polypeptides and N-acetyl-neuraminic acid increased the binding of most *B. glabrata* polypeptides to zymosan. The gel was stained with silver nitrate. Molecular weight (MW) markers are in kilodaltons.



Galactose or glucosamine had no effect on the binding capabilities of plasma to zymosan.

3.6. Two-Dimensional Electrophoresis and Immunoblotting.

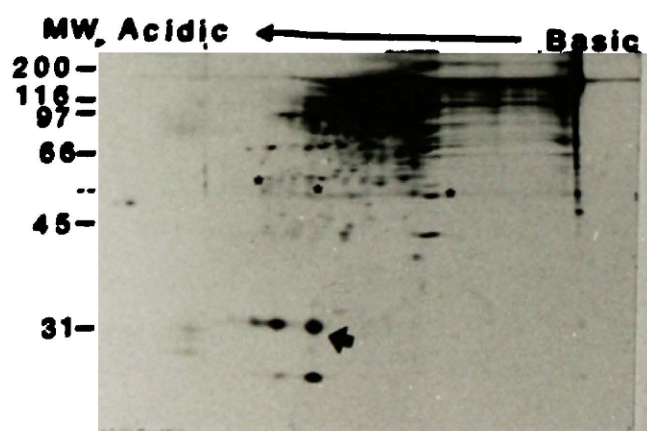
Two-dimensional electrophoresis revealed three groups of proteins in the molecular weight range of 55 kDa exist in the plasma of M-line, 13-16-R1 and 10-R2 *B. glabrata* (Fig. 7A, 8A, 9A, respectively). Two polypeptides of approximately 55 kDa exist in the basic region of all plasma samples. A protein of 55 kDa in the middle of the pI range and an acidic protein of approximately 55 kDa was also present (Fig 7B, C, D; Fig 8A, B, C; Fig. 9A, B, C).

Antibodies to M-line, 10-R2 and 13-16-R1 plasma recognized the 55 kDa region in all strains. Proteins of 55 kDa formed a string of polypeptides across two-dimensional separated 13-16-R1 *B. glabrata* plasma probed with anti-R2 (Fig. 8D).

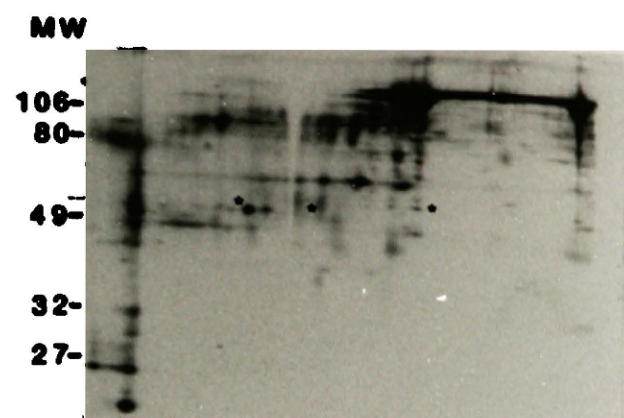
Polypeptides of 30 and 25 kDa from the acidic region, and polypeptides of approximately 40 kDa, ranging from basic to acidic, often were not present and thus could not react with the antibodies. Further repetitions of this experiment indicated that these 25, 30, and 40 kDa polypeptides were often present in both silver stained gels and immunoblots. When present on the immunoblots of M-line *B. glabrata* they appeared similar to those polypeptides from 10-R2 and 13-16-R1 *B. glabrata* (data not shown). Plasma polypeptides from 13-16-R1 *B. glabrata* also were occasionally absent, i.e. polypeptides of approximately 40 kDa with a pI from basic to acidic (Fig. 8B, C). When the 40 kDa polypeptides appeared on the 13-16-R1 plasma immunoblots, polypeptides from the basic end were missing.

Figure 7. Two-dimensional electrophoresis of plasma from M-line *B. glabrata*. (A) The isoelectric focused plasma was subjected to SDS-PAGE (12%) and stained with silver nitrate. Note the three groups of polypeptides in the molecular weight range of 55 kDa indicated with stars and the polypeptides in the 32 kDa region indicated by an arrow. Molecular weight (MW) markers are in kilodaltons. For B, C and D, M-line isoelectric focused plasma was subjected to SDS-PAGE (12%), electroblotted to a membrane, and probed with antibodies to 10-R2 *B. glabrata* plasma (anti-R2) (B), antibodies to M-line plasma (anti-M) (C) and, antibodies to 13-16-R1 *B. glabrata* plasma (anti-R1) (D). Note the presence of the 55 kDa polypeptides and the absence of the 32 kDa regions. Molecular weight (MW) markers in kilodaltons, for C and D, are the same as depicted in B.

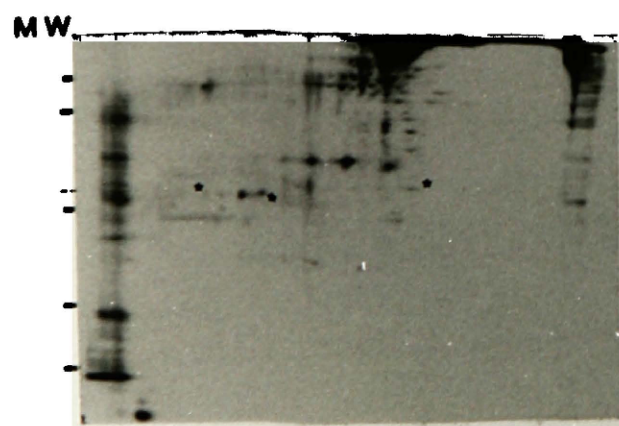
A.



B.



C.



D.

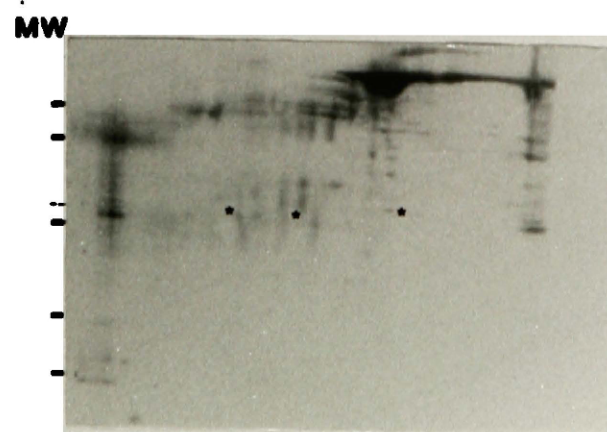
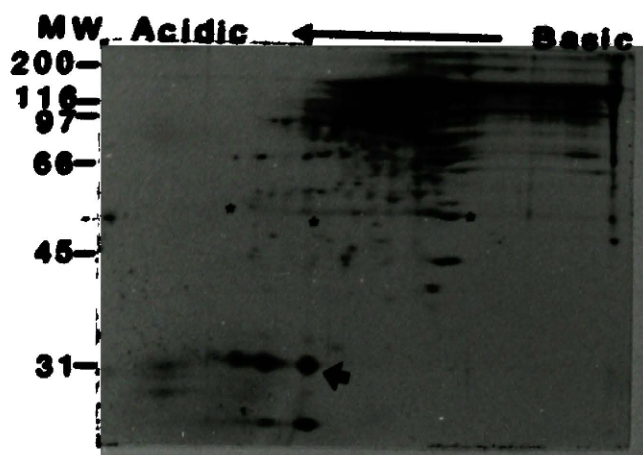


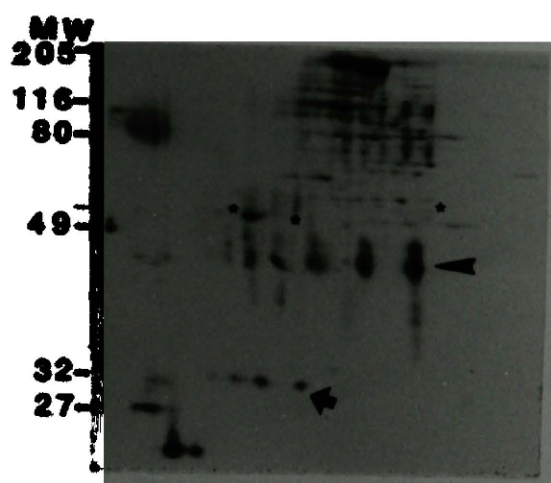
Figure 8. Two-dimensional electrophoresis of plasma from 13-16-R1 *B. glabrata*.

(A) The isoelectric focused plasma was subjected to SDS-PAGE (12%) and stained with silver nitrate. Note the polypeptides in the molecular weight range of 55 kDa indicated with stars and the polypeptides in the 32 kDa range indicated with an arrow. Molecular weight (MW) markers are in kilodaltons. For B, C and D 13-16-R1 isoelectric focused plasma was subjected to SDS-PAGE (12%), electroblotted to a membrane, and probed with antibodies to 10-R2 *B. glabrata* plasma (anti-R2) (B), antibodies to M-line *B. glabrata* plasma (anti-M) (C) and, antibodies to 13-16-R1 plasma (anti-R1) (D). Note the three groups of 55 kDa proteins indicated with stars, the 40 kDa proteins indicated with an arrowhead and the 32 kDa proteins indicated with and arrow. Molecular weight (MW) markers in kilodaltons, for C and D, are the same as depicted in B.

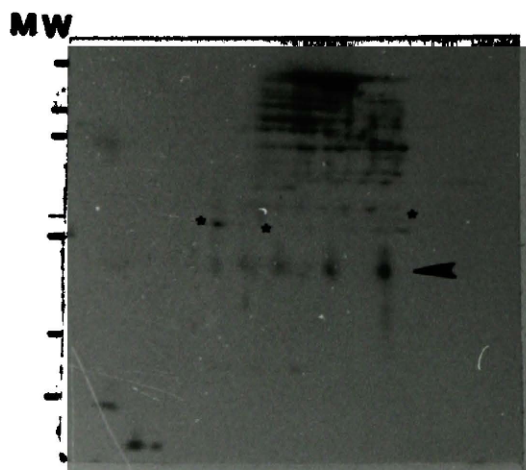
A.



B.



C.



D.

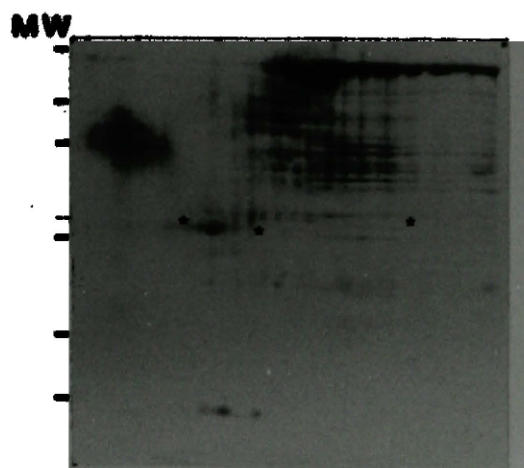
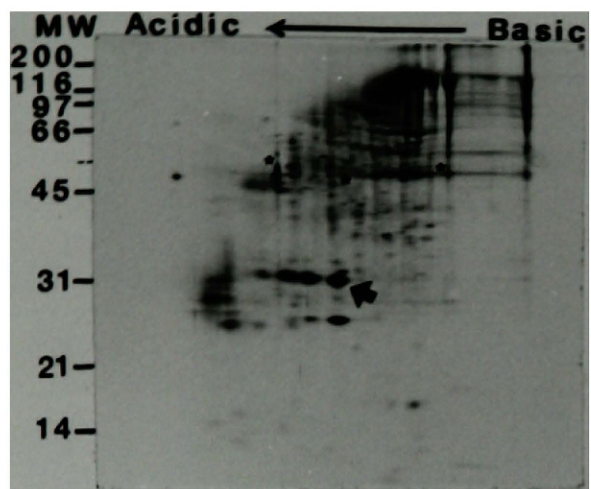
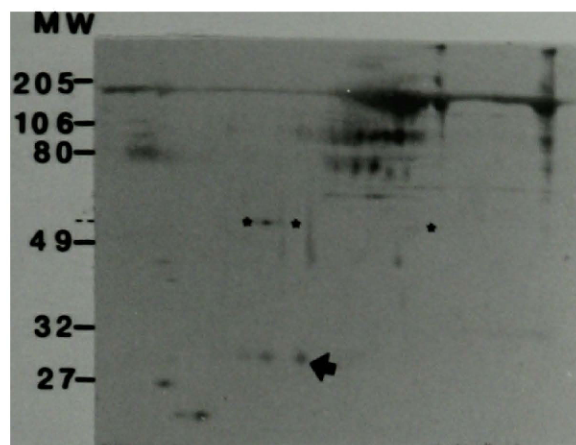


Figure 9. Two-dimensional electrophoresis of plasma from 10-R2 *B. glabrata*. Note the polypeptides in the molecular weight range of 55 kDa are indicated with stars and the polypeptides in the 32 kDa region indicated with an arrow. (A) *B. glabrata* (10-R2) plasma was isoelectric focused, subjected to SDS-PAGE (12%) and stained with silver nitrate. Molecular weight (MW) markers are in kilodaltons. For B, C and D 10-R2 isoelectric focused plasma was subjected to SDS-PAGE (12%), electroblotted to a membrane, and probed with antibodies to 10-R2 plasma (anti-R2) (B), antibodies to M-line plasma (anti-M) (C) and, antibodies to 13-16-R1 *B. glabrata* (D). Molecular weight (MW) markers in kilodaltons, for C and D, are the same as depicted in B.

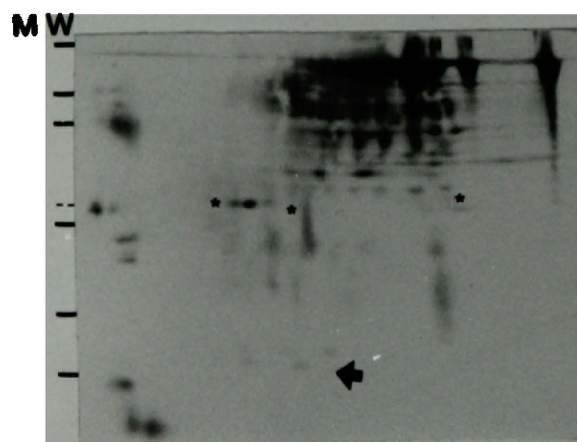
A.



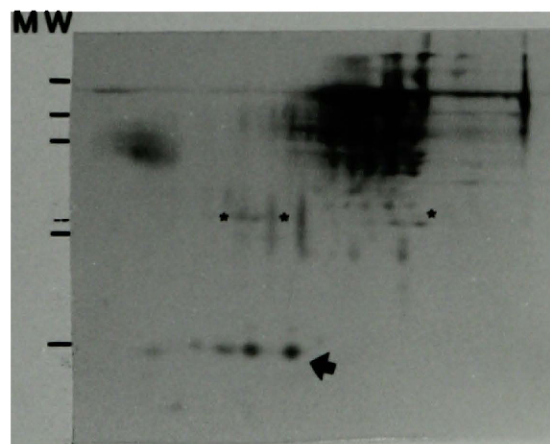
B.



C.



D.



Chapter 4

Discussion

A 55 kDa plasma polypeptide(s) from schistosome-resistant (10-R2) *B. glabrata* has been shown to bind to living sporocysts (Spray and Granath, 1990). Based on this observation, this thesis took five different approaches to characterize the 55 kDa polypeptide(s) from schistosome-susceptible (M-line) and resistant (13-16-R1, 10-R2) strains of *B. glabrata*. The first was to determine whether the 55 kDa polypeptide(s) from resistant *B. glabrata* bound to chemically-fixed sporocysts. Second, the antigenic characteristics of the 55 kDa plasma polypeptide(s) from M-line, 13-16-R1 and 10-R2 *B. glabrata* was examined by generating antibodies to this isolated polypeptide(s) and using these reagents for immunoblot analyses. Third, the effect carbohydrates have on the ability of the 55 kDa and other plasma polypeptides to bind to zymosan was examined. Fourth, antibodies to M-line plasma were used to immunoprecipitate M-line, 13-16-R1 and 10-R2 plasma to determine whether the 55 kDa or other plasma polypeptides were unique to 13-16-R1 and 10-R2 snails. Fifth, plasma was subjected to two-dimensional electrophoresis, electroblotted to a membrane, and probed with antibodies to determine whether there was more than one antigenic subunit of the 55 kDa polypeptide.

The goal of the first set of experiments was to determine which radiolabeled polypeptides of 13-16-R1 *B. glabrata* plasma bound to chemically-fixed *S. mansoni* sporocysts. This experiment was based partly on the results of Loker *et al.* (1984) who found that schistosome-resistant (13-16-R1, 10-R2) *B. glabrata* plasma

agglutinated both fixed and living *S. mansoni* sporocysts whereas schistosome-susceptible (M-line) *B. glabrata* plasma only agglutinated live sporocysts. These experiments indicated that schistosome-resistant plasma possessed polypeptides with sporocyst binding specificity which caused the agglutination of fixed sporocysts. Subsequent studies using ^{125}I -labeled *B. glabrata* plasma identified a 55 kDa polypeptide unique to schistosome-resistant (10-R2) *B. glabrata* that bound to living sporocysts (Spray and Granath, 1990). This finding led Spray and Granath (1990) to hypothesize that the unique 55 kDa polypeptide from 10-R2 plasma may agglutinate fixed sporocysts. It was also hypothesized that when the 55 kDa polypeptide was bound to sporocysts it may act as a receptor or as an opsonin for hemocytes. Fryer and Bayne (1989) showed that a protein of a similar molecular weight (57 kDa) was an opsonin, *in vitro*, for yeast. Subsequent studies have shown, *in vitro*, that other *B. glabrata* plasma components were opsonins for latex beads (Uchikawa and Loker, 1992), and when calcium ions were absent *B. glabrata* plasma lectins were required for *in vitro* phagocytosis of sheep red blood cells (Zelck and Becker, 1992). To be an opsonin for fixed sporocysts a polypeptide must be able to bind to the parasite, such as the lectins that have been identified which bind to sporocysts (Zelck and Becker, 1990). These lectins do not exist in *B. glabrata* plasma and to date the only *B. glabrata* polypeptide with sporocyst specificity is the 55 kDa polypeptide from 10-R2 snails. Therefore, before it can be determined if the 55 kDa from schistosome-resistant 13-16-R1 *B. glabrata* agglutinates, opsonizes or acts as a receptor on fixed sporocysts it was determined whether this polypeptide bound to fixed sporocysts.

Several difficulties exist in comparing the data in Fig. 1 to those from the study conducted by Spray and Granath (1990). In the present study, data for polypeptides from 13-16-R1 was obtained whereas Spray and Granath (1990) provided information for M-line and 10-R2 *B. glabrata*. It is also possible that M-line plasma possesses polypeptides in the 55 kDa region that would have adhered to fixed sporocysts in this assay. However, Spray and Granath (1990) showed that M-line plasma did not possess a 55 kDa polypeptide with the ability to bind to living sporocyst. A second difficulty is that Spray and Granath (1990) found living *S. mansoni* sporocysts to acquire 10-R2 *B. glabrata* plasma polypeptides of 210, 180, 116 and 55 kDa whereas in this study, living sporocysts acquired 13-16-R1 *B. glabrata* plasma polypeptides of 210, 66 and 55 kDa. Further, 13-16-R1 and 10-R2 *B. glabrata* are different snail strains which may complicate generalizations about properties of resistant *B. glabrata*. In spite of these difficulties both 13-16-R1 and 10-R2 are schistosome-resistant, plasma of both agglutinate *S. mansoni* sporocysts and both have the unique 55 kDa polypeptide that adhere to living sporocysts. Thus, results presented in this first series of experiments of the thesis were evaluated using the hypothesis of Spray and Granath (1990).

The results of incubating glutaraldehyde-fixed and living parasites with equal counts per min of a ^{125}I -labeled plasma sample from 13-16-R1 *B. glabrata* showed that three polypeptides (210, 66, 55 kDa) bound to both fixed and living parasites (Fig. 1). When the parasites were fixed, however, more of the 210 and less of the 55 kDa polypeptide bound. This decrease in binding of the 55 kDa polypeptide appeared

equivalent to the increased amount of 210 kDa polypeptide that bound. An explanation for this result is that living sporocysts excrete proteinase(s) which cleaved the 55 kDa polypeptide(s) from the 210 kDa polypeptide. However, when plasma was incubated with fixed sporocysts no proteinase(s) would be present to cleave the 55 kDa polypeptide from the 210 kDa polypeptide.

Support for live parasites possessing such proteinases comes from the finding that miracidia, the stage of the parasite that transforms to sporocysts, were shown to contain neutral exopeptidase and leucine aminopeptidase (Bogitsh, 1983; Xu and Dresden, 1986). More significantly, cysteine proteinase(s) active on high molecular weight snail plasma proteins were detected in the culture medium when miracidia transformed to sporocysts (Lodes and Yoshino, 1993). These proteinase(s) recognized substrates containing arginine in the P1 position and the hydrophobic amino acids (leu, phe) at P2. If such a proteinase(s) was responsible for the results in this thesis it would be expected that the proteinase(s) secreted from live sporocysts would cleave the 55 kDa from the 210 kDa polypeptide. For fixed sporocysts it would be expected that such a proteinase(s) would not be secreted and, therefore, less of the 55 kDa polypeptide would be produced to bind to sporocysts. This expectation offers an explanation for the results which showed that less of the 55 kDa was bound to fixed sporocysts (Fig. 1). Hence, it seems likely that the quantity of the 55 kDa polypeptide that bound to living or fixed *S. mansoni* sporocysts was dependent upon the enzymatic action of the proteinase(s) identified by Lodes and Yoshino (1993).

The presence or absence of certain parasitic enzymes could also have affected the results of an experiment performed by Loker *et al.* (1989). They found that when *E. paraensei* redia and sporocysts were fixed, hemocyte adherence was significantly increased as compared to when the parasite was alive. When using fixed parasites the proteinase(s) and other components which may aid in degrading hemocyte membrane proteins would be absent. This absence may have allowed hemocyte adherence to increase.

Other enzymes exist which may influence the results of experiments comparing fixed and living parasites. For example, cercariae have recently been shown to possess a phospholipase specific for glycosylphosphatidylinositol (Hawn and Strand, 1993). Living sporocysts may also possess phospholipase and this enzyme may allow the sporocyst to alter specific regions of its tegument. The absence of such phospholipases from fixed sporocysts may have affected the outcome of experiments performed in this thesis and by those conducted by Loker *et al.* (1989).

Outside of enzymatic cleavage, there are three alternative explanations for why fixed and living sporocysts obtained different quantities of the 210 and 55 kDa polypeptides. One explanation is that the 210 kDa polypeptide that bound to fixed sporocysts may not have been completely solubilized. Thus, an analysis of this sample by SDS-PAGE would show more of this polypeptide to be present. Incomplete solubilization has previously been demonstrated with *B. glabrata* plasma (Fig. 2; Spray, 1989). The 55 kDa polypeptide(s) from three strains of *B. glabrata* was excised from a polyacrylamide gel, re-solubilized, and re-subjected to SDS-

PAGE (Fig. 2). This gel indicated that the 55 kDa polypeptide disassociated to many smaller molecular weight proteins but it did not re-aggregate to form higher molecular weight proteins. Spray (1989) also showed that when plasma from M-line *B. glabrata* was separated by SDS-PAGE, cut out of the gel and re-subjected to SDS-PAGE, many of the proteins disassociated to lower molecular weight polypeptides of which the 55 kDa polypeptide was often the only disassociation product. Apparently, complete solubilization was not achieved in the two former experiments. In the present study, it is possible that incomplete solubilization of the 210 and 55 kDa polypeptides was responsible for the quantitative differences in polypeptide adherence to fixed and living parasites (Fig. 1). However, this seems unlikely because polypeptides that bound to sporocysts were solubilized in a higher ratio of solubilization buffer to protein than in the previously discussed studies. In addition, both samples of polypeptides that bound to fixed and living sporocysts were treated with the same solubilization buffer and boiled for the same amount of time. As the solubilization buffer used was denaturing and reducing the 55 kDa subunit would have to be associated with the 210 kDa molecule by forces other than hydrogen-bonds and disulfide linkages.

A second explanation for the observed quantitative differences in polypeptide acquisition between living and fixed sporocysts is that the 210 and 55 kDa polypeptides had different affinities for fixed sporocysts. Glutaraldehyde-fixation, which cross-links proteins, may have altered the surface structure of the sporocysts tegument. These alterations may have caused the 55 kDa polypeptide to have a lower

affinity for the sporocysts and the 210 kDa polypeptide to have a higher affinity. Support for certain polypeptides having different affinities for the surface of fixed sporocyst comes from Lodes and Yoshino (1993). They found that M-line and 10-R2 *B. glabrata* hemocytes synthesized two polypeptides (19 and 46 kDa) that adhered to living sporocysts. However, when sporocysts were fixed, less of the 19 kDa polypeptide and none of the 46 kDa polypeptide adhered. The reasons for these quantitative and qualitative differences between living and fixed parasites is unknown. Since Lodes and Yoshino (1993) only washed their sporocyst polypeptide samples two times, a decrease in affinity of the 19 and 46 kDa polypeptides for the fixed sporocysts could account for their results. In the current study, sporocyst-¹²⁵I-plasma mixtures were washed with sPBS until the radioactive counts stabilized (usually more than ten times). After extensive washing the polypeptides that remained bound to sporocysts had a high affinity. Possibly, the 210 kDa polypeptide had a higher affinity for the tegument of fixed sporocysts while the 55 kDa polypeptide had a decreased affinity. This possibility is not favored since both fixed and living parasite-polypeptide samples were washed an equal number of times.

A third explanation for the observed quantitative differences in polypeptide acquisition to fixed sporocysts (Fig. 1) is that when fixed, parasites may no longer be able to interfere with the attachment, shedding, or modulation of the 210 kDa polypeptide. Support for living sporocysts interfering with the immune components of snails was presented by Fryer and Bayne (1990). They found that once *S. mansoni* had infected M-line *B. glabrata*, a reduction in hemocyte phagocytosis occurred in the

presence of plasma, but not in its absence. The interference was found to be directed at hemocytes. Although not noted by Fryer and Bayne (1990) it is apparent that this interference must have been mediated through components in snail plasma. When Lodes and Yoshino (1993) incubated fixed sporocysts with hemocyte secreted polypeptides the binding of a 19 kDa polypeptide was decreased and a 46 kDa polypeptide did not bind. These investigators took the proper precautions of incubating the sporocysts and plasma at 4°C in the presence of sodium azide (Dunn and Yoshino, 1991) to prevent fixed sporocysts from shedding or altering acquired polypeptides. Nevertheless, the authors speculated that fixed sporocysts could have shed or modulated acquired polypeptides. The main problem with this speculation is that fixed sporocysts have no metabolic capacity to shed or modulate bound plasma components. An additional problem with this speculation is that for a control, sporocyst ES products were pre-incubated with the hemocyte-secreted polypeptides for 24 hr before the addition to sporocysts but no effect on polypeptide binding was observed. It appears that if ES products affect polypeptide acquisition to sporocysts the living sporocysts must be present to provide unknown factors that facilitate the action of the ES products.

Additional studies on sporocyst-polypeptide interactions showed that living sporocysts acquired *B. glabrata* polypeptides from both schistosome-resistant and susceptible *B. glabrata* (Bayne *et al.*, 1986; Spray and Granath, 1990; Lodes and Yoshino 1993). Bayne *et al.* (1986) used polyclonal antibodies generated to hemoglobin-depleted plasma of schistosome-susceptible (M-line) and resistant (10-R2)

B. glabrata to determine, *in vitro*, if M-line and/or 10-R2 *B. glabrata* plasma proteins would bind to sporocysts. Unique polypeptides were not detected, but different quantities of polypeptides from both snail strains bound to the living sporocysts. Since Spray and Granath (1989) showed that M-line and 10-R2 *B. glabrata* plasma only had subtle differences in polypeptide profiles, these authors indicated that the method used by Bayne *et al.* (1986) actually detected the commonality between M-line and 10-R2 *B. glabrata*.

In contrast to Bayne *et al.* (1986), experiments in this thesis have identified specific polypeptides from schistosome-resistant *B. glabrata* that adhered to sporocysts, with quantities differing between fixed and living parasites. The drastic decrease in the amount of the 55 kDa polypeptide that bound fixed sporocysts (Fig. 1) makes it unlikely that, as an isolated protein, this polypeptide would be responsible for the agglutination of fixed sporocysts as hypothesized by Spray and Granath (1990). It is possible that the 55 kDa polypeptide(s) remains associated with the 210 kDa polypeptide in the absence of living sporocyst proteinase(s). When the 210 kDa polypeptide is associated, the 55 kDa subunit may mediate agglutination of fixed sporocysts. Further study is warranted since the 55 kDa polypeptide was present on the sporocyst tegument only after exposure to ES products. During penetration by a miracidium, a schistosome-resistant snail may respond to a parasite proteinase(s) by allowing cleavage of the 55 kDa polypeptide from the 210 kDa polypeptide. Schistosome-susceptible snails may not possess the 55 kDa polypeptide with sporocyst binding capabilities. To determine whether a biologically active 55 kDa is produced

by the proteinase(s), parasite proteinase(s) could be isolated as described by Lodes and Yoshino (1993), incubated with the isolated *B. glabrata* 210 kDa polypeptide and then, by SDS-PAGE analysis, determine whether the 55 kDa is an end product. When bound to the sporocyst it could be determined whether these 210 and 55 kDa polypeptides act as receptors or as opsonins for hemocytes. A sporocyst opsonin or recognition factor would be significant since molluscs have been shown to possess plasma recognition factors (Anderson and Good, 1976; Dikkeboom *et al.*, 1985; Fryer *et al.*, 1989), but none of these factors have been shown to bind to sporocysts. The factor(s) determining if a protein binds to sporocyst may be dependent upon the antigenic characteristic of the polypeptide.

A second series of experiments in this thesis studied the antigenic characteristics of the 55 kDa polypeptide(s) from M-line, 13-16-R1 and 10-R2 *B. glabrata* by raising antibodies to these polypeptides. Results indicated that antibodies to the 55 kDa plasma protein from M-line snails recognized at least one common epitope in the majority of the plasma proteins from M-line, 10-R2 and 13-16-R1 *B. glabrata*. The most obvious explanation for the cross-reactivity was that the antigen injected into the rabbits included traces of other proteins. This explanation is not favored since the 55 kDa range was purified twice at two different percentages of acrylamide. It is possible that traces of polypeptides in the 55 kDa range were present in the antigen injected into the rabbits, but certainly not all of the polypeptides in *B. glabrata* plasma were present in the antigen. The anti-55 kDa antibodies reacted with proteins

greater than 200 kDa and it is highly unlikely that proteins of this size would deviously migrate to 55 kDa at two different acrylamide percentages.

An explanation for why antibodies generated to a single *B. glabrata* plasma polypeptide reacted with the majority of plasma polypeptides is that most of the plasma components of *B. glabrata* are hemoglobin subunits or breakdown products (Granath *et al.*, 1987). Confirmation of *B. glabrata* plasma proteins consisting mainly of hemoglobin was obtained by Granath (1988). Using a heme-stain, the majority of polypeptides in *B. glabrata* were shown to be hemoglobin or hemoglobin subunits. It is interesting that polypeptides of approximately 50 kDa in hemoglobin-depleted samples of plasma did not react with the heme stain. Using peptide mapping, Spray and Granath (1989) also showed that five isolated polypeptides from plasma of schistosome-resistant and susceptible strains of *B. glabrata* were structurally homologous to the hemoglobin molecule. In fact, antibodies to isolated hemoglobin reacted with the majority of *B. glabrata* plasma polypeptides separated by HPLC (Granath *et al.*, 1987). These results confirm the hypothesis of Yoshino (1983) that anti-*B. glabrata* plasma antibodies react with hemoglobin-like determinates on the membrane of *B. glabrata* hemocytes.

Other investigators have reported that antibodies to *B. glabrata* plasma cross-react with sporocysts, *B. glabrata* hemocytes, and *B. glabrata* plasma components (Bayne *et al.*, 1987; Boswell *et al.*, 1987; Granath *et al.*, 1987; Granath and Aspevig 1993; Yoshino, 1983). Antibodies generated to the parasite cross-reacted with both *B. glabrata* hemocytes (Abdul-Salam and Michelson, 1983) and plasma from patentlly

infected *B. glabrata* (Loker *et al.*, 1986). It is apparent that epitopes common to many components in this host/parasite system exist. The molecular nature of these cross-reactive epitopes between *B. glabrata* and *S. mansoni* were shown to be carbohydrate associated molecules (Dissous *et al.*, 1986).

Bayne *et al.* (1987) also found antibodies to a purified *B. glabrata* plasma component to cross-react with the majority of *B. glabrata* plasma components. These authors were unable to achieve isolation of specific antibodies from polyvalent anti-sera. Therefore, a different method, which used *B. glabrata* plasma proteins to remove the cross-reactive antibodies, was performed in this thesis to remove the cross-reactivity from anti-55 kDa sera. While removing the cross-reactivity plasma polypeptides in the 47 and 84 kDa regions from M-line, 13-16-R1 and 10-R2 *B. glabrata* did not react with anti-M-55 (Fig. 3B), hence these polypeptides probably possessed either fewer of the common epitopes or different epitopes for which the antibodies had idiotypes. Cross-reactive antibodies to the 55 kDa polypeptide(s) probably recognized carbohydrate epitopes since Dissous *et al.* (1986) showed that antibodies made to components in this host-parasite system were specific to carbohydrates. Because of this, cross-reactivity removed from anti-sera to the 55 kDa polypeptide(s) probably removed antibodies specific for the dominant carbohydrates. Possessing less of a common carbohydrate is a more likely explanation because when immunoblots were counterstained to confirm protein transfer, the 47 and 84 kDa polypeptides appeared equal in quantity to polypeptides that were recognized by the

antibodies. Also, SDS-PAGE analysis of *B. glabrata* plasma showed that these 47 and 84 kDa polypeptide regions appeared equal in quantities between snail strains.

It is apparent that anti-M-55 was specific for the 55 kDa polypeptide(s) from M-line and 10-R2 *B. glabrata* plasma, but not for the 55 kDa polypeptide(s) from 13-16-R1 *B. glabrata* (Fig. 4A). It is unlikely that the failure of anti-M-55 to recognize the 55 kDa polypeptide(s) from 13-16-R1 *B. glabrata* was due to a large number of unique amino acid sequences for the following reasons: 1) antibodies generated to the 55 kDa polypeptide(s) from 13-16-R1 (anti-R1-55) recognized the 55 kDa polypeptide(s) from the other two *B. glabrata* strains (Fig. 4B); 2) these three *B. glabrata* strains are closely related (Richards, 1975a, b); 3) the 55 kDa polypeptide(s) from schistosome-susceptible M-line and schistosome-resistant 10-R2 *B. glabrata* were found to share structural homology when analyzed by two-dimensional peptide mapping (Spray and Granath, 1989). A more likely possibility for this lack of recognition (Fig. 4A) is that the 55 kDa polypeptide(s) from 13-16-R1 snails has fewer or altogether different cross-reactive carbohydrate molecules to which anti-M-55 could bind. Support for the 55 kDa polypeptide(s) from 13-16-R1 *B. glabrata* having fewer carbohydrate molecules is that anti-R1-55 reacted stronger to the 55 kDa polypeptide(s) from M-line and 10-R2 *B. glabrata* than to the 55 kDa polypeptide(s) from 13-16-R1 (Fig. 4B). Support for different carbohydrates molecules comes from the finding that a *B. glabrata* plasma polypeptide was shown to be recognized by two separate lectins with different carbohydrate specificities (Vietri and Granath, 1992). Thus if a protein was no longer recognized by the remaining

antibodies, the protein either had fewer or less of the common carbohydrate for which these antibodies were specific.

The 55 kDa polypeptide(s) from both schistosome-resistant (10-R2) and schistosome-susceptible (M-line) snails was recognized by anti-M-55 and anti-R1-55. Therefore, the common carbohydrate molecules that may have served as epitopes probably are not involved with the sporocyst binding capabilities of the 55 kDa polypeptide(s) from 10-R2 plasma. This conclusion would be specific for the particular carbohydrate molecule(s) associated with the 55 kDa polypeptide since carbohydrate molecules are complex and appear to be important in the parasite-hemocyste interactions. In fact, anti-sporocyst antibodies interfered with hemocyste adherence (Bayne *et al.*, 1984) and these antibodies probably were binding to carbohydrates.

Due to the finding that antibodies raised to either schistosome larvae or *B. glabrata* plasma were cross-reactive with *B. glabrata* plasma, an attempt was made to isolate specific antibodies from polyclonal anti-sera (Bayne *et al.*, 1987). A specific antibody would be indicative of more than one type of antigenic region serving as an epitope for the antibodies. As previously mentioned, Bayne *et al.* (1987) attempted to isolate specific antibodies from antisera generated to *B. glabrata* plasma proteins using several methods, but these were very different from the technique used in this study. Initially, their experiments utilized anti-sera raised to a *B. glabrata* hemagglutinin (anti-HA). Anti-HA was incubated with plasma from *B. glabrata* lacking the hemagglutinin but all of the antibodies were precipitated. Next, rabbit red

blood cells were used to remove all of the hemagglutinin activity from M-line plasma and then this plasma was used to immunoprecipitate anti-HA. Once again, all antibodies were depleted from anti-HA. Next, antibody to 10-R2 *B. glabrata* plasma was incubated with M-line plasma that was bound to a solid phase (CNBr-sepharose) matrix to try and isolate a strain-specific antibody. Once again all antibodies were depleted. In their last effort the plasma hemagglutinin was separated by SDS-PAGE, blotted and incubated in anti-HA. The antibodies that had bound to the HA were removed, isolated and tested for specificity to the hemagglutinin in 10-R2 and M-line plasma. Antibody specificity was not achieved; cross-reactivity still existed. These investigators thus concluded that a single polypeptide carried epitopes shared by the majority of plasma proteins. In addition to the above, the data from experiments in this thesis indicate, for the first time, that an antibody specific to a *B. glabrata* plasma component can be isolated from a polyclonal mixture and that some plasma components have multiple sites that serve as common epitopes.

In a third series of experiments a method was developed to assess the effect *B. glabrata* carbohydrate binding polypeptides on phagocytosis by snail hemocytes. Carbohydrate binding polypeptides (agglutinins/lectins) have been implicated as factors influencing phagocytosis by *B. glabrata* hemocytes (Loker *et al.*, 1989). Zymosan is a particle commonly used to monitor phagocytosis by snail hemocytes. *B. glabrata* plasma was found to adhere to zymosan so it was determined if these plasma components could be inhibited to binding to zymosan by preincubating with a sugar (Fig. 6). When preincubated, the sugar should saturate carbohydrate binding

receptors and inhibit the polypeptides from binding to zymosan (Fig. 6). When plasma was incubated with mannan 180, 116, and 55 kDa polypeptides no longer bound to zymosan. Galactose and glucosamine had no effect on polypeptide binding. Interestingly, when N-acetyl-neuraminic acid was present more plasma components bound to zymosan than when particles were not preincubated with a sugar (Fig. 6). This sugar evidently acts as a bridge to enable plasma components to bind to zymosan. It is unknown if this sugar is naturally found in *B. glabrata* plasma or if it is involved in this host/parasite interaction, but more investigation seems warranted on the effects of sugars on plasma. In addition, the effect of specific *B. glabrata* polypeptides on hemocyte phagocytosis needs to be evaluated.

In the fourth series of experiments an immunoprecipitation assay was performed. Purified IgG that was generated to M-line plasma (anti-M) was incubated with plasma from M-line, 13-16-R1 and 10-R2 *B. glabrata*. Protein A was then used to pellet the antibody/plasma complexes and the supernatant that possibly contained unique antigens was analyzed by SDS-PAGE. Any plasma protein(s) removed from the supernatant of schistosome-susceptible (M-line), but left in the supernatant of schistosome-resistant plasma (13-16-R1 or 10-R2), would identify the protein as antigenically unique. Upon first analysis, most *B. glabrata* proteins remained in the supernatant therefore more antibodies were added to the unsolubilized supernatant (data not shown). After the second immunoprecipitation it is evident that all plasma proteins were still present in the M-line *B. glabrata* supernatant sample except a 41 kDa polypeptide (Fig. 5). Hence, this 41 kDa polypeptide from schistosome-resistant

snails appears to be antigenically unique and not removed by anti-M. To clearly illustrate that the 41 kDa polypeptide was absent, 1.5 times as much protein from the M-line supernatant, as compared to the other supernatants, was examined on SDS-PAGE. Anti-M may not of had paratopes and/or it may not have had a strong enough avidity for this 41 kDa polypeptide from resistant snails. Further, excessive quantities of the 41 kDa polypeptide normally found in resistant snail plasma does not appear to be responsible for this polypeptide not being immunoprecipitated. This is shown by the finding that M-line and 10-R2 plasma not immunoprecipitated show that 10-R2 and M-line plasma have relatively equal quantities of the 41 kDa polypeptide (Fig. 5). In other words, there was not such an excess of the 41 kDa polypeptide in 10-R2 plasma that anti-M did not contain enough antibodies to remove it.

In this immunoprecipitation experiment results indicated that most plasma proteins were not removed by anti-M (Fig. 5). Why plasma proteins were not removed from M-line *B. glabrata*, the snail plasma against which the antibodies were generated, is unclear. Presumably, even though anti-M was added in excess there evidently was not enough antibody to bind to the quantitatively dominant plasma component. Granath *et al.* (1987) showed that the majority of *B glabrata* plasma proteins were hemoglobin and hemoglobin subunits. Support for a lack of hemoglobin-specific antibodies comes from the fact that antibodies are generated to the most antigenic components, and not components that are in the greatest quantity. Thus, anti-M had a mixture of antibodies that recognized the most antigenic component(s) which may not have been the component(s) that was present in the

largest quantity in *B. glabrata* plasma. Therefore, the ratio of anti-M to plasma used probably did not contain enough antibodies specific for hemoglobin. Alternatively, the proteins may not have been removed because the proteins were aggregated together and did not come in contact with the antibodies. Support for snail plasma proteins sticking together comes from Granath *et al.* (1987). When they subjected plasma to HPLC nearly all the fractions which were analyzed by SDS-PAGE, gave polypeptide profiles similar to whole plasma. Consequently, even if proteins were not sticking together, any polypeptides remaining in the supernatant may have given a polypeptide profile similar to the supernatant samples (Fig. 5). Additional evidence indicating that a polypeptide can break down to a smaller molecular weight polypeptide is that an isolated 55 kDa polypeptide(s) from the three strains of *B. glabrata* broke down to many smaller molecular weight polypeptides, but it is evident that the 41 kDa polypeptide was not one of the breakdown products (Fig. 2). Likewise, Spray (1989) showed that most plasma polypeptides from *B. glabrata* broke-down to many smaller molecular weight polypeptides when separated on SDS-PAGE a second time but, once again the 41 kDa polypeptide was not one of the breakdown products. In addition, it was shown by Granath *et al.* (1987) that a band of approximately 41 kDa was present in a hemoglobin-depleted plasma fraction from M-line *B. glabrata*. From the above it appears the 41 kDa polypeptide from 13-16-R1 or 10-R2 *B. glabrata* is unique to schistosomeresistant snails and does not appear to be a breakdown product of any polypeptides present in *B. glabrata* plasma.

A polypeptide of approximately 41 kDa was shown to be synthesized and secreted from M-line and 10-R2 hemocytes cultured *in vitro* (Lodes and Yoshino, 1993). However, although not noted by these authors, this 41 kDa polypeptide appeared to have been secreted in greater quantities by hemocytes if the hemocytes were allowed to adhere to sporocysts. Quantitative responses have been implicated as meaningful responses determining host/parasite compatibility (Lodes and Yoshino, 1993). In addition, proteins not associated with hemoglobin, such as the 41 kDa, may be important in determining the ability of a snail to clear a schistosome infection (Bayne, 1981; Granath *et al.*, 1987). Furthermore, since hemocytes from schistosome-resistant snails can be cytotoxic to sporocysts *in vitro* without the aid of plasma factors (Bayne *et al.*, 1980a), this antigenically unique 41 kDa polypeptide originating from hemocytes may facilitate schistosome destruction.

The fifth set of experiments in this thesis involved two-dimensional electrophoresis of M-line, 13-16-R1 and 10-R2 *B. glabrata* plasma which was then probed with antibodies to M-line, 13-16-R1 and 10-R2 *B. glabrata* plasma to determine whether the 55 kDa or any other polypeptides were unique to schistosome-resistant snails. All combinations of two-dimensional separated plasma probed with the different antibodies revealed two pairs of basic proteins in the 55 kDa molecular weight range. Spray and Granath (1990) believed that the upper one-half of the 55 kDa range in 10-R2 plasma, as shown by SDS-PAGE, may have been the protein that adhered to *S. mansoni* sporocysts *in vitro*. It is noteworthy that by SDS-PAGE the isoelectric point is not determined. In this study the upper 55 kDa band is present in

M-line plasma as well as in 10-R2 and 13-16-R1 plasma. A protein of 55 kDa in the middle of the pI range and an acidic protein of approximately 55 kDa was present in addition to the doublet at the basic end (Fig. 6B, C, D; Fig. 7A, B, C; Fig. 8A, B, C). Proteins of approximately 55 kDa formed a string of polypeptides from basic to acidic (Fig. 7D). Antibodies to M-line, 10-R2 and 13-16-R1 plasma reacted similarly to all 55 kDa polypeptides from all strains. Since at least one of the 55 kDa polypeptides from schistosome-resistant snails has the unique property of binding to primary sporocysts it must be different from the 55 kDa polypeptide of schistosome-susceptible snails. However, it is evident from this experiment that polyclonal antibodies cannot detect this uniqueness. The antibodies probably recognized the common carbohydrate epitope previously discussed. It can be concluded that all of the 55 kDa polypeptides possess the common epitope.

The similarities in recognition of the 55 kDa does not negate its importance. As discussed earlier in this thesis the 55 kDa subunit that is unique to schistosome-resistant snails may not be present until it is cleaved by a sporocyst proteinase(s). Thus, the unique 55 kDa polypeptide may not be present since the plasma was not exposed to ES products. If the 55 kDa subunit is a unique cleavage product of hemoglobin it would be difficult to detect by probing with polyclonal antibodies. Hemoglobin is a large antigenic molecule with similar multiple subunits between snail strains. Thus a polyclonal mixture raised to the 55 kDa polypeptide also would have many antibodies for the hemoglobin molecules. This would mask possible uniqueness of the hemoglobin molecule.

When M-line *B. glabrata* plasma was isoelectric focused, separated on SDS-PAGE, blotted onto a nylon membrane and probed with homologous and heterologous antibodies to snail plasma, polypeptides of 30 and 25 kDa from the acidic region and polypeptides of approximately 40 kDa, ranging from basic to acidic, often did not react with the antibodies. Counterstaining which has a sensitivity of 80 ng on nylon membranes, did not detect the polypeptides. However, repetitions of this experiment indicated that these polypeptides were present and recognized by the antibodies. These evasive polypeptides, when present on immunoblots of M-line plasma appeared similar to those present on immunoblots of 10-R2 and 13-16-R1 *B. glabrata* (data not shown). The factors which determine the absence or presence of these polypeptides obviously were not elucidated. Since pooled samples were used the factor determining the absence or presence of these polypeptides must have been constant enough to affect all of the samples uniformly.

Plasma polypeptides of approximately 40 kDa, with a pI of basic to acidic, from 13-16-R1 plasma also were occasionally absent (Fig. 7B, C). The presence of these 40 kDa polypeptides seems to correlate with a decreased quantity of hemoglobin. Conditions for why the hemoglobin appeared to disassociate to these 40 kDa polypeptides is unknown. Oddly, when the 40 kDa polypeptides appeared in immunoblots of 13-16-R1 plasma, polypeptides from the basic end were missing. It is possible that the factor(s) which determines whether a protein is basic is the same factor which allows the 40 kDa polypeptide to be detected in this two-dimensional system.

The absence of specific polypeptides by two-dimensional electrophoresis analysis could be due to *in vivo* post-translational modifications (e.g. methylation, phosphorylation, acetylation, sulfation). If a *B. glabrata* plasma polypeptide was modified in such a way that it was more basic than a pH of 7 it would not have entered in the first dimension. Alternatively, if a polypeptide was modified in such a way that it was more acidic than a pH of 5 it would have run all the way through the first dimension. It is apparent that some polypeptides were modified in such a way that they only entered the basic portion of the gel and were unable to isoelectric focus (Fig. 6; Fig. 7D; Fig. 8). When subjected to the second dimension (SDS-PAGE) these proteins at the basic tip of the gel appeared to have been associated with hemoglobin. On occasion, polypeptides were absent from the acidic ends (Fig. 6; Fig. 7C, D; Fig. 8C). These missing proteins either had a different pI which prevented their presence in the first dimension or they had a different molecular weight so that they would migrate to a different location.

It is known that methylation and phosphorylation are modifications which are reversible whereas acetylation is not (Alberts, *et al.*, 1983). The modifications may have been the former two, for which the variables determining reversibility were not accounted. Changes in pI or molecular weight of a polypeptide could be the result of carbohydrates that were associated with the polypeptide. Glycoproteins often display heterogeneity (Spiro, 1973) and will not be resolved as a single charged species when isoelectric focused (Dunbar, 1987). Many *B. glabrata* plasma proteins possess carbohydrates (Vietri and Granath, 1992). In fact, most plasma proteins are believed

to possess at least one common carbohydrate molecule. Support for this is that monoclonal and anti-plasma antibodies recognized most *B. glabrata* plasma proteins only when the carbohydrate was present on the proteins (Dissous *et al.*, 1986). Further, glycosylation can effect the binding of SDS to a protein (Dunbar, 1987). If a protein is differentially glycosylated the quantities of SDS that can bind varies. Although most SDS is removed in the first dimension to allow a protein to reach its pI, trace amounts may remain. The amount and degree of remaining SDS could affect the rate at which the protein reaches its pI (Dunbar, 1987). Length of time protein samples were run was not found to be a significant factor affecting the presence or absence of a protein. Since SDS was present in the solubilization buffer, any modification that affected the hydrogen-bonding capabilities of polypeptides would not be responsible for the presence or absence of a polypeptide.

The nutritional status of the snail may determine the presence of a polypeptide by two-dimensional electrophoresis. Snails starved up to 24 hrs consistently had the 25 and 30 kDa acidic polypeptides, but plasma obtained from fed snails also often had the same polypeptide profile. Thus it appears that these 25 and 30 kDa polypeptides are not polypeptides involved with the nutritional status of a snail.

Conclusions.

This thesis took five approaches to characterize plasma factors from *B. glabrata* that may play a role in resistance to the human blood fluke *S. mansoni*. A 55 kDa plasma polypeptide(s) from schistosome-resistant (13-16-R1) *B. glabrata* was found to bind to living sporocysts but, when chemically-fixed, much less of this polypeptide

bound (Fig. 1). This polypeptide is not thought to be responsible for the agglutination of fixed sporocysts as proposed by Spray and Granath (1990). Further, it was speculated that a proteinase(s) produced by living sporocysts may cleave the 55 kDa from the 210 kDa polypeptide. This hypothesis could be tested by incubating the 210 kDa plasma polypeptide from *B. glabrata* with sporocyst proteinase(s) to determine if the 55 kDa polypeptide is a product. If it is a product it could be used to determine if the 55 kDa polypeptide acts as an opsonin or receptor on sporocysts for hemocytes as proposed by Spray and Granath (1990). The second series of experiments revealed that antibodies to the purified 55 kDa polypeptide(s) from M-line, 13-16-R1 and 10-R2 *B. glabrata* were highly cross-reactive with the majority of *B. glabrata* plasma proteins. Cross-reactivity was removed by incubating the anti-55 kDa sera with *B. glabrata* plasma that did not contain the 55 kDa protein(s). While removing cross-reactivity certain proteins were found to possess more of a common epitope than other proteins (Fig. 3). The anti-55 antibodies were probably specific for common carbohydrate molecules found in all *B. glabrata* plasma proteins. Determining the role of glycosylated plasma polypeptides in invertebrate immunity will undoubtedly aid in a better understanding of schistosome-snail interactions. When cross-reactivity was removed from anti-M-55 and anti-R1-55, antibodies reacted stronger to the 55 kDa polypeptide from M-line and 10-R2 than to the 55 kDa polypeptide from 13-16-R1 *B. glabrata* (Fig. 4). Since the 55 kDa protein from 10-R2 and 13-16-R1 have the ability to bind to sporocysts it is speculated that the portion of this protein recognized by the antibodies is not involved in binding to sporocysts. In a third

series of experiments *B. glabrata* plasma polypeptides were found to bind to zymosan particles and the binding of specific polypeptides were inhibited if the particles were preincubated with specific carbohydrates. Interestingly, the sugar N-acetyl-neuraminic acid resulted in most *B. glabrata* plasma components adhering to zymosan. This method of allowing or preventing carbohydrate specific polypeptides from binding to zymosan could be utilized to assess the affect of *B. glabrata* plasma proteins on zymosan-phagocytosis by snail hemocytes. An immunoprecipitation experiment to remove the common antigens between schistosome-susceptible and resistant snails revealed that a 41 kDa polypeptide was removed from the plasma of susceptible snails, but not from the plasma of resistant snails. Therefore, this polypeptide is antigenically unique to resistant snails. Future techniques to characterize this 41 kDa polypeptide from susceptible and resistant snails could include: isolation, purification, peptide mapping, phagocytosis assays, cytokine assays, sequencing, etc. Lastly, two-dimensional electrophoresis revealed that 3 groups of proteins exist in the 55 kDa molecular weight range in *B. glabrata*. Repeating the plasma/sporocyst incubation experiments would reveal exactly which one of these polypeptides bind to sporocysts. In addition, determining the factors responsible for the presence or absence of polypeptides in the 25 and 30 kDa range may reveal information about the function of these polypeptides. Performing experiments of the type listed above will contribute further to a better understanding of *B. glabrata*-schistosome immunobiology.

Chapter 5

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